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(71) Applicant: TARGETECH, INC. [US/US]; 290 Pratt Street, Meridien, CT 06450 (US).

(72) Inventors: PLOURDE, Robert, Jr.; Apartment 1305, 871 Stevens Avenue, Solana Beach, CA 92075 (US). CARMICHAEL, Ellen; 103 Richmont Lane, West Hartford, CT 06117 (US). SPITALNY, George, L.; 6 Brookfield Court, Cheshire, CT 06410 (US). FINDEIS, Mark, A.; 57 West Cedar Street, #10, Boston, MA 02114 (US). ERNST, Michael, F.; 1019 Eastern Pt. Road, Grotten, CT 06340 (US). ROBINSON, Brett; 51 Fox Hill Lane, Enfield, CT 06082 (US).

(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

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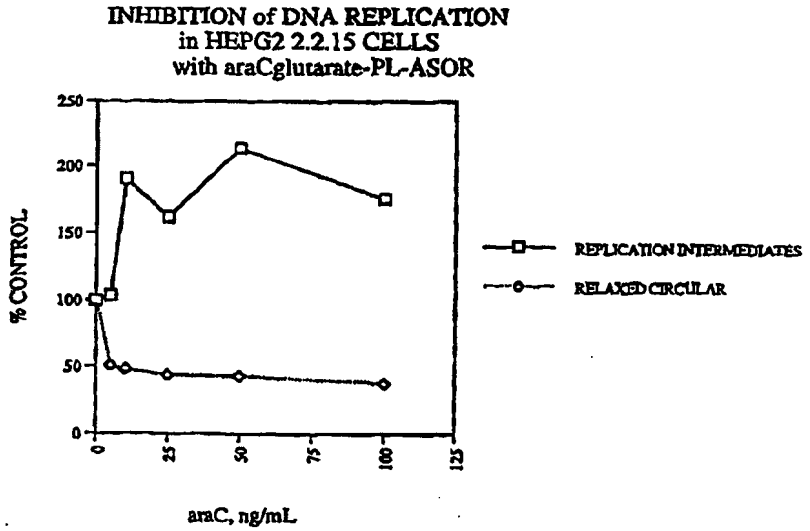

(57) Abstract

The invention provides conjugates for targeting a therapeutic agent to a cell with asialoglycoprotein receptors. The conjugates comprise a therapeutic agent and ligand for the asialoglycoprotein receptor, wherein the therapeutic agent and the ligand are linked by a bridging agent. The bridging agent can be a crosslinker, a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule. In a preferred embodiment, the therapeutic agent is a nucleoside analog or colchicine and the ligand is asialoorosomucoid, arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)-glutamate. Preferred crosslinkers include aminoacyl derivatives, carboxyacyl derivatives, phosphate, peptides and reductively-labile crosslinkers. Preferred polyfunctional carrier molecules include polyamino acids and polysaccharides. The conjugates of the invention can be used to target a therapeutic agent to a cell, for example to inhibit viral DNA replication in a virally-infected hepatocyte.

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HEPATOCYTE-TARGETED DRUG CONJUGATES

Background of the Invention

Nonspecific toxicity is a major impediment to the development of chemotherapeutic agents for the treatment of diseases such as viral infections and cancer. Since the molecular mechanisms by which both of these disease types propagate are intimately associated with normal cellular metabolism, the discovery of drugs which selectively block propagation of these diseases has been slow. Thus, conventional chemotherapy relies on the balanced use of therapeutic agents, many of which have a narrow range of active concentrations before toxicity is manifested in uninfected or nontransformed cells, thereby prohibiting the use of greater concentrations of the therapeutic agent. Additional impediments to the efficacy of certain therapeutic agents include inactivation of the agents within the body and/or rapid excretion of the agents from the body, thus limiting their therapeutic activity.

An approach that has been taken to increase the therapeutic activity of a drug has been to conjugate the drug to a macromolecule which acts as a carrier for the drug. Conjugation of a drug to a macromolecule can slow the rate of drug excretion and increase cellular uptake of the drug, presumably by non-specific pinocytosis. Certain drugs have been conjugated to polymeric macromolecules such as polysaccharides and polyamino acids, resulting in decreased inactivation of the drug and/or decreased excretion of the drug from the body. See for example, Bernstein, A., et al., (1978) *J. Natl. Cancer Inst.* 60:379-384; Kato, Y., et al., (1984) *Cancer Res.* 44:25-30; Kéry, V., et al., (1990) *Int. J. Biochem.* 22:1203-1207; Onishi, H., et al., (1991) *Drug Design and Delivery* 7:139-145.

However, conjugation of a drug to a macromolecule which does not function as a ligand for a specific receptor does not address the problem of non-specific toxicity of therapeutic agents. One general strategy for increasing the specificity of therapeutic agents involves attachment of a drug to a cell-specific ligand to effect targeted delivery of the drug to a desired cell population. Conjugation of a drug to a ligand which binds to a structure on the surface of a cell to be targeted for drug delivery (e.g., a virally-infected or malignant cell) can increase the specific uptake of the drug by the target cell via receptor-mediated endocytosis and reduce non-specific cytotoxicity.

One type of cell-specific ligand which has been used for drug conjugation is a monoclonal antibody directed against a surface structure present on a target cell. For example, targeted delivery of therapeutic agents to tumor cells by conjugating the agents to

an anti-tumor cell antibody has been investigated extensively (for a review see Pietersz, G.A. (1990) *Bioconjugate Chemistry* 1(2):89-95).

Another type of cell-specific ligand which has been used for drug conjugation is a glycoprotein which binds to a membrane receptor for the glycoprotein present on a target cell (for a review see Bodmer, J.L. and Dean, R.T. (1988) *Methods in Enzymology*, 112:298-306). One target cell which is of particular clinical importance is the parenchymal liver cell, or hepatocyte, which is a primary site of infection for hepatitis viruses, such as hepatitis B virus. The nucleoside analogs adenine arabinofuranoside monophosphate (*ara*AMP) and acyclovir monophosphate (ACVMP) have shown promise as therapeutic agents for treatment of hepatitis B virus (HBV), but their use as free drugs has been associated with problems such as toxicity, rapid clearance (for *ara*AMP) and poor cellular uptake (for ACVMP) (see for example Jacyna, M.R. and Thomas, H.C. (1990) *British Medical Bulletin*, 46:368-382; Sacks, S.L., et al. (1979) *JAMA* 241:28; Whitley, R., et al. (1980) *Drugs* 20:267; Balfour, H.H. (1984) *Ann. Rev. Med.* 35:279; Weller, I.V.D., et al., (1983) *J. Antimicrob. Chemother.* 11:223). *Ara*AMP and ACVMP have been targeted to liver cells by conjugating them to lactosaminated human serum albumin (hereinafter L-HSA) which binds to the asialoglycoprotein receptor present on liver cells (Fiume, L., et al. (1981) *FEBS Letters* 129:261-264; Fiume, L., et al. (1988) *Pharm. Acta Helv.* 63:137-139; Fiume, L., (1989) *Naturwissenschaften* 76:74-76; U.S. Patent No. 4,725,672; U.S. Patent No. 4,794,170). In these cases, the phosphate moiety was used to crosslink the nucleoside analogs to L-HSA. ACVMP has also been conjugated to L-HSA via a glutarate crosslinker or a succinate crosslinker (U.S. Patent No. 4,725,672). A conjugate of *ara*AMP coupled to L-HSA was selectively cleared from circulation by the asialoglycoprotein receptor and inhibited DNA synthesis in hepatocytes (Fiume, L., et al. (1981) *FEBS Letters* 129:261-264). A conjugate of ACVMP coupled to L-HSA was shown to release the free drug in liver cells (Fiume, L., (1989) *Naturwissenschaften* 76:74-76). Both conjugates lowered woodchuck hepatitis virus DNA levels at doses lower than the unconjugated drugs (Ponzetto, A., et al. (1991) *Hepatology* 14:16-24) and the *ara*AMP-L-HSA conjugate inhibited HBV replication in humans (Fiume, L., et al. (1988) *Lancet* 2:13-15). However, there can be drawbacks to using lactosaminated albumin as a cell-specific ligand. The specificity of L-HSA for the asialoglycoprotein receptor results from the galactosyl residues that are attached to the albumin upon lactosamination. If this process is inefficient, the number of galactosyl residues which are coupled to the albumin may not be sufficient to produce a ligand with high affinity for the asialoglycoprotein receptor, thereby reducing the targeting ability of the conjugate.

Polyfunctional carrier molecules have also been used to increase the therapeutic activity of drugs. Polyfunctional carrier molecule have a multiplicity of reactive

side chains to which other molecules can be conjugated and thus have the advantage that many small molecules can be coupled to a single molecule of carrier. Drugs have been conjugated to polyfunctional carrier molecules, such as polyglutamic acid, to increase the non-specific uptake of the drug (see for example Kato, Y., et al., (1984) *Cancer Res.* 44:25-30). However, attempts at combining the use of a polyfunctional carrier molecule and a cell-specific ligand to target a therapeutic agent have shown limitations. In one study (Fiume, L. et al. (1986) *FEBS Letters* 203:203-206), polylysine was used in conjugates for targeting antiviral agents to virus-infected hepatocytes through the ASGR by coupling galactose residues to polylysine. *Ara*AMP and ACVMP were then conjugated to the galactosyl-polylysine. Of the two constructs, only *ara*AMP-galactosyl-polylysine effectively targeted hepatocytes and inhibited DNA synthesis in *Ectromelia* virus-infected mice; the ACVMP-galactosyl-polylysine conjugate was inactive.

Summary of the Invention

This invention pertains to drug conjugates which can target a therapeutic agent to a cell which expresses asialoglycoprotein receptors. The therapeutic agent is targeted to the asialoglycoprotein receptor by conjugating it to a ligand for the asialoglycoprotein receptor. The therapeutic agent is conjugated to the ligand by one or more bridging agents which function to couple the therapeutic agent and the ligand. The bridging agent(s) has the property that it allows the therapeutic agent and the ligand to be coupled without destroying the therapeutic activity of the agent or the binding activity of the ligand. Binding of the ligand to the asialoglycoprotein receptor facilitates uptake of the conjugate by the cell via receptor-mediated endocytosis.

In a preferred embodiment, the conjugate comprises a therapeutic agent and a ligand for the asialoglycoprotein receptor selected from asialoorosomucoid, arabinogalactan and the synthetic ligand YEE(GalNAcAH)$_3$. The therapeutic agent is conjugated to the ligand via a bridging agent which can be a crosslinker, a polyfunctional carrier molecule or both a crosslinker and a polyfunctional carrier molecule. When a crosslinker is used alone, the crosslinker covalently binds to both the therapeutic agent and the ligand, thereby coupling the therapeutic agent to the ligand. For example, crosslinkers which react with amino groups, carboxyl groups or sulfhydryl groups on the ligand can be used. Similarly, when a polyfunctional carrier molecule is used alone, the polyfunctional carrier molecule covalently binds to both the therapeutic agent and the ligand, thereby coupling the therapeutic agent to the ligand. For example, a polyfunctional carrier molecule with reactive aldehyde groups, such as polyaldehyde dextran, can be used. Alternatively, when both a crosslinker and a polyfunctional carrier molecule are used, the reagents are chosen such that the crosslinker covalently binds to both the therapeutic agent and the polyfunctional carrier molecule, and

the polyfunctional carrier molecule covalently binds to the ligand, thereby coupling the therapeutic agent to the ligand. The crosslinker can be coupled to the polyfunctional carrier molecule by an amide bond, a phosphoamide bond or a disulfide bond. For example, an aminoacyl crosslinker can be used with a polyfunctional carrier molecule having multiple reactive carboxyl groups, or alternatively, a carboxyacyl crosslinker can be used with a polyfunctional carrier molecule having multiple reactive amino groups.

Preferred crosslinkers for use in the conjugates include phosphate derivatives, carboxyacyl derivatives of succinate and glutarate, aminoacyl derivatives of trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate, a derivative of (3-(2-pyridyldithio)propionate or a peptide comprising an amino acid sequence Leu-Ala-Leu. Preferred polyfunctional carrier molecules include polyamino acids, such a polylysine, polyornithine, polyglutamic acid and polyaspartic acid and polysaccharides such as polyaldehyde dextran.

The conjugates of the invention can be used to target a therapeutic agent to hepatocytes, which express asialoglycoprotein receptors. Therapeutic agents which are effective against viral infections of hepatocytes can thus be conjugated to a ligand for the asialoglycoprotein receptor. For example, antiviral drugs effective against a hepatitis virus, such as hepatitis B virus, can be used in the conjugates. A preferred type of antiviral therapeutic agent is a nucleoside analog. The invention encompasses conjugates comprising a nucleoside analog and an asialoglycoprotein receptor ligand, such as asialoorosomucoid, including conjugates wherein the nucleoside analog is 9-β-D-arabinofuranosylcytosine, 9-(2-hydroxyethyoxymethyl)guanine, dideoxycytidine, 9-β-D-arabinofuranosyladenine and 3'-azido-3'-deoxythymidine. The conjugates of the invention are useful for inhibiting viral DNA replication in virally-infected cells, such as hepatitis B virus-infected hepatocytes.

The invention further provides methods for targeting a therapeutic agent to a cell expressing asialoglycoprotein receptors in a subject. The method involves forming a conjugate of the therapeutic agent and an asialoglycoprotein receptor ligand, such as asialoorosomucoid, and administering the conjugate in a physiologically acceptable vehicle to the subject.

Brief Descriptions of the Drawings

Figure 1 is a graph depicting the effect of increasing concentrations of an *ara*C-glutarate-PL-ASOR conjugate on the intracellular accumulation of replication intermediate (RI) and relaxed circular (RC) forms of HBV DNA in HBV DNA-transfected 2.2.15 cells.

Figure 2 is a graph depicting the effect of increasing concentrations of free ACV and an ACVMP-PL-ASOR conjugate on the intracellular accumulation of relaxed circular HBV DNA in HBV DNA-transfected 2.2.15 cells.

Figure 3 is a graph depicting the effect of increasing concentrations of free ACV and an ACVMP-PL-ASOR conjugate on the extracellular accumulation of HBV DNA in the culture medium from HBV DNA-transfected 2.2.15 cells.

Figure 4 is a graph depicting the effect of increasing concentrations of a ddC-PAD-ASOR conjugate on the intracellular accumulation of relaxed circular HBV DNA in HBV DNA-transfected 2.2.15 cells.

Detailed Description of the Invention

The invention relates to conjugates which can target a therapeutic agent to a cell expressing asialoglycoprotein receptors. The therapeutic agent is targeted to the asialoglycoprotein receptor (hereinafter ASGR) by conjugating it to a ligand for ASGR. The ligand serves to target the therapeutic agent to a cell with asialoglycoprotein receptors and to facilitate uptake of the conjugate by the cell via receptor-mediated endocytosis. Because the conjugates of the invention achieve the effect of targeting the therapeutic agent to a cell, a lower dosage of a conjugate is needed to achieve a desired therapeutic effect compared to the unconjugated therapeutic agent. Additionally, because the conjugates are directed away from cells which do not express the asialoglycoprotein receptor it is to be expected that the non-specific cytotoxicity of the therapeutic agent will be decreased *in vivo*.

The term "conjugate" is intended to include two or more molecular species which are covalently bonded to each other. The conjugates of the invention are composed of at least a therapeutic agent and a ligand for ASGR, and usually at least one additional molecular species which functions as a bridging agent between the therapeutic agent and the ligand. Thus, there are essentially three components of the conjugate to be considered: the therapeutic agent, the ligand and the means by which the two are conjugated together (i.e., the bridging agent(s)), which will be discussed in more detail in the sections below.

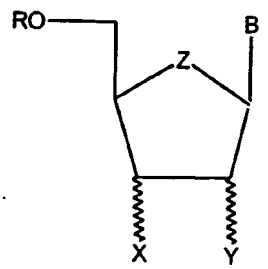
I. Therapeutic agents

The term "therapeutic agent" is intended to include molecules which are administered to a subject with the intent of changing, in a beneficial way, a physiological

function in the subject or with the intent of treating, in a beneficial way, a disease or disorder in the subject. Therapeutic agents include drugs, conventional antiviral agents (including nucleoside analogs), conventional anti-tumor agents, reverse transcriptase inhibitors, topoisomerase I inhibitors, topoisomerase II inhibitors, prokaryotic DNA gyrase inhibitors, DNA binding agents, hormones, growth factors, vitamins, proteins and peptides and analogs thereof, nucleic acids and analogs thereof, and other bioactive molecules. For example, the therapeutic agent can be an antiviral drug which is targeted to virally-infected cells which express ASGR as a means of treating the viral infection.

A preferred ASGR-expressing cell type to which therapeutic agents are targeted is a hepatocyte. Thus, an antiviral drug which is effective against a viral infection of hepatocytes can be conjugated to a ligand for ASGR to target the antiviral drug to virally-infected hepatocytes. A viral infection of hepatocytes can be due to infection by any hepatotropic virus. Examples of hepatotropic viruses include hepatitis virus A, hepatitis virus B, hepatitis virus C and hepatitis virus D. A preferred hepatotropic virus against which a therapeutic agent is directed is hepatitis B virus.

One therapeutic approach to treating viral infections, such as hepatitis B virus, is to use a drug which interferes with viral DNA synthesis, such as a nucleoside analog. For example, two nucleoside analogs, 9-β-D-arabinofuranosyladenine (*ara*A) and 9-(2-hydroxyethyoxymethyl)guanine (also known as acyclovir; ACV), have been tested in patients with chronic hepatitis B virus infection (Hoofnagle, J.H. et al. (1984) *Gastroenterology* 86:150-157; Weller, I.V.D., et al., (1985) *Gut* 26:745-751; Sacks, S.L., et al., (1979) *JAMA* 241:28; Weller, I.V.D., et al., (1983) *J. Antimicrob. Chemother.* 11:223-231; Alexander, G.J.M., et al., (1986) *J. Hepatol.* 3(Suppl. 2):S123-S127). While the free nucleoside analogs displayed a blocking effect on viral growth, dose-related side-effects were observed. Incorporation of a nucleoside analog into a conjugate of the invention can increase the therapeutic activity of the drug, thereby decreasing the dosage of the drug necessary for therapeutic effectiveness. Accordingly, in one embodiment, the therapeutic agent of the conjugate is a nucleoside analog. As used herein, the term "nucleoside analog" is intended to include molecules having the general formula:



wherein Z is oxygen, sulfur or carbon, B is a nucleoside base or analog, X and Y are substituent groups such as OH, H, N_3, F etc. and R is a functional group that allows attachment of the nucleoside analog to a crosslinker and/or carrier molecule by a covalent bond. Examples of suitable functional groups include those that provide a free -OH, $-NH_2$, -COOH or -SH moiety.

The term "nucleoside analog" is also intended to include acyclic nucleosides of the general formula:

wherein B is a nucleoside base or analog, Z is oxygen, sulfur or carbon, and R is a functional group that allows attachment of the nucleoside analog to a crosslinker and/or carrier molecule by a covalent bond. Examples of suitable functional groups include those that provide a free -OH, $-NH_2$, -COOH or -SH moiety. Examples of acyclic nucleotides which are effective antiviral agents which can be used in the conjugates of the invention are described in U.S. Patent No. 4,199,574 by Schaeffer.

Preferred nucleoside analogs for use in conjugates of the invention include 9-β-D-arabinofuranosyladenine (*ara*A), 9-β-D-arabinofuranosylcytosine (*ara*C), 2',3'-dideoxycytidine (ddC) and 3'-azido-3'-deoxythymidine (AZT). A preferred acyclic nucleoside analog is 9-(2-hydroxyethyoxymethyl)guanine (ACV). Other possible nucleoside analogs include gancyclovir, famcyclovir, pencyclovir, bromovinyldeoxyuridine, phosphonoformate, the 2',3'-dideoxynucleosides of adenosine (ddA), inosine (ddI), guanosine (ddG), thymidine (ddT) and uracil (ddU), 9-β-D-arabinofuranosyladenine-erythro-9-(2-hydroxynonyl)adenine (AraA-EHNA), 2'-fluoro-1-β-D-arabinofuranourosyl-5-methyluracil (FMAU), 2'-fluoro-1-β-D-arabinofuranourosyl-5-ethyluracil (FEAU), 2'-fluoro-1-β-D-arabinofuranosyl-5-iodouracil (FIAU), 2'-fluoro-1-β-D-arabinofuranosyl-5-iodocytidine (FIAC), 3'-fluoro-ddC, 5-chloro-ddC, 3'-fluoro-5-chloro-ddC, 3'-azido-5-chloro-ddC, 3'-fluoro-ddT, 3'-fluoro-ddU, 3'-fluoro-5-chloro-ddU, 3'-azido-ddU, 3'-azido-5-chloro-ddU, 2'-6'-diaminopurine 2', 3'-dideoxyriboside (ddDAPR) and a carbocylic analog of deoxyguanosine (2'-CDG).

In addition to nucleoside analogs, other types of therapeutic agents can be used to inhibit viral infections, such as hepatitis virus infections. For example, reverse transcriptase inhibitors, topoisomerase inhibitors, gyrase inhibitors and DNA binding agents have been shown to inhibit hepatitis B virus DNA replication (Civitico, G., et al. (1990) *J.*

Med. Virol. 31:90-97). Therapeutically effective compounds which can be used as therapeutic agents in the conjugates of the invention include the topoisomerase II inhibitors ellipticine, amsacrine, adriamycin and mitrozantrone, the prokaryotic DNA gyrase inhibitor coumermycin A1 and the DNA binding agents neocarzinostatin and chloroquine (which either intercalate or nick DNA).

II. Ligands for the Asialoglycoprotein Receptor

A therapeutic agent is targeted to a cell expressing ASGR by conjugating it to a ligand for the asialoglycoprotein receptor (also referred to as the hepatic Gal/GalNAc-specific receptor). The term "a ligand for the asialoglycoprotein receptor" is intended to include any molecule which binds to the asialoglycoprotein receptor. One type of ligand for the asialoglycoprotein receptor is an asialoglycoprotein with clustered terminal galactose residues. Such an asialoglycoprotein can be prepared from sialic acid-terminating glycoproteins with penultimate galactosyl residues. The galactose residues are exposed by desialation of the glycoprotein using standard techniques. For example, glycoproteins can be desialated by treating them with the enzyme neuraminidase. Alternatively, glycoproteins can be desialated by acid hydrolysis as described in Example 1. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin, asialoceruloplasmin, asialohaptoglobulin and desialylated vesicular stomatitis virus. Orosomucoid, fetuin, ceruloplasmin and haptoglobulin can be obtained from blood plasma and then desialated.

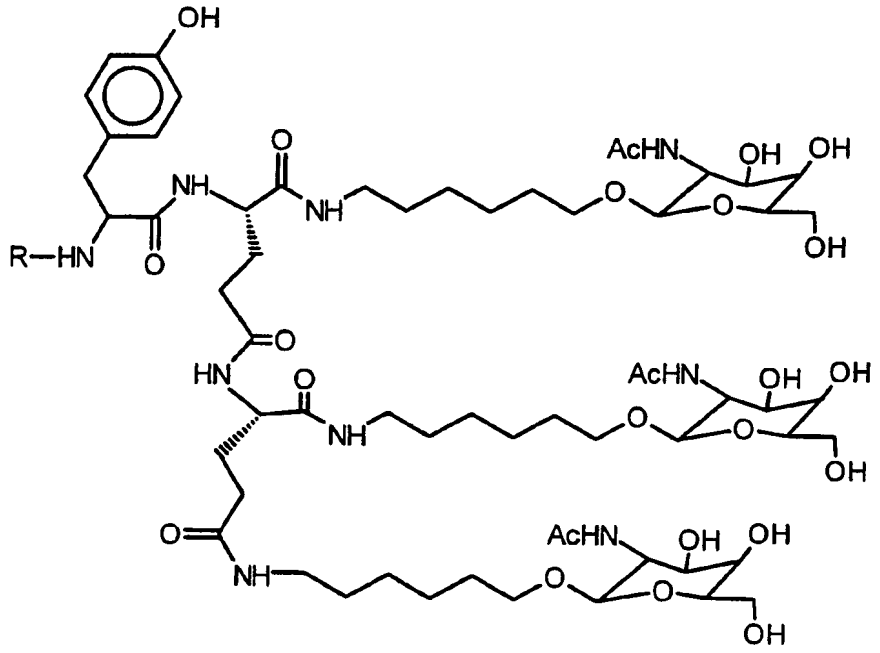
A preferred ASGR ligand for use in the conjugates of the invention is the asialoglycoprotein asialoorosomucoid (hereinafter ASOR). It should also be appreciated that certain alterations (e.g., amino acid deletions or point mutations) of the orosomucoid protein, or derivatives of the protein or attached carbohydrate moiety, can be made without destroying the ability of the glycoprotein to bind to ASGR. Such altered or derivatized forms of ASOR are intended to be within the scope of the term "asialoorosomucoid" as used herein. ASOR can be prepared from orosomucoid (also referred to as α-1-acid glycoprotein), isolated from human plasma, by desialation of the isolated orosomucoid to expose penultimate galactose groups (such as described in Example 1). It has been found that when several radiolabeled plasma-derived asialoglycoproteins are coinjected *in vivo* into the circulation, asialoorosomucoid is cleared most rapidly from the circulation, indicating that ASOR is taken up rapidly by the liver (see *J. Biol. Chem.* (1970) 245:4397; and PCT Application WO 92/22310). Additionally, ASOR is rich in carboxylic acid groups which allow for coupling of therapeutic agents or bridging agents to ASOR through these groups.

Another type of ligand for ASGR is a neoglycoprotein, a protein which has been modified to be a ligand for ASGR. For example, terminal galactosyl residues can be

coupled to a protein to convert it to a ligand for ASGR. For example, galactose-terminal carbohydrates, such as lactose, can be coupled to a protein by reductive amination.

Another type of ligand which can be used in the conjugates of the invention is a carbohydrate which binds to ASGR. For example, a polysaccharide with terminal galactose residues can be used to target a therapeutic agent to ASGR. A preferred carbohydrate ligand is arabinogalactan. Arabinogalactan is a component of the cell walls of many species of trees and plants. Structurally, arabinogalactan consists of a galactose backbone with branch chains of arabinose and galactose. Generally, the ratio of galactose to arabinose is between 5:1 and 10:1 (see Glickman, ed. (1982) *Food Hydrocolloids*, CRC Press). Derivatives of arabinogalactan can be prepared which provide functional groups that allow attachment of arabinogalactan to a therapeutic agent, a crosslinker or a polyfunctional carrier molecule. For example, an amino derivative or a carboxyl derivative of arabinogalactan can be used to prepare a conjugate of the invention in which arabinogalactan serves as the ligand for ASGR.

Another type of ligand which can be used in the conjugates of the invention is a synthetic ligand for ASGR. The synthetic ligand comprises a carbohydrate moiety having a binding specificity for ASGR linked to a peptide via an amide bond. A preferred carbohydrate moiety is N-acetylgalactosamine. For example, two or more carbohydrate moieties can be linked to a di- or tri-peptide to form a cluster ligand specific for ASGR. The synthetic ligand also comprises an organic structure having a functional group available for forming a covalent bond with a therapeutic agent, a crosslinker or a polyfunctional carrier molecule. A preferred synthetic ligand is the Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate, referred to herein as YEE(GalNAcAH)$_3$. YEE(GalNAcAH)$_3$ can be synthesized as described in Lee et al. (1987) *Glycoconjugate Journal* 3:317, or as described in U.S. Patent Application Serial No. 08/045,985 by Findeis et al., the contents of which are hereby incorporated by reference. The structure of YEE(GalNAcAH)$_3$ can be represented by the following formula:



wherein R is H or $COCH_2CH_2CO_2H$.

III. Conjugation of the Therapeutic Agent to the ASGR Ligand: Bridging Agents

A therapeutic agent is conjugated to a ligand for the asialoglycoprotein receptor by means of an intermediary which functions as a bridging agent to connect the therapeutic agent to the ligand. The bridging agent must allow for coupling of the therapeutic agent to the ligand without destroying either the therapeutic activity of the therapeutic agent or the binding activity of the ligand and should be stable in the circulation *in vivo*. Additionally, the bridging agent preferably should allow for release of the therapeutic agent intracellulary in an active, functional form, although this may not be an absolute requirement. The bridging agent and conjugation mechanism are critical components of the conjugate that can play a major role in determining whether a conjugate is active or not (i.e., a conjugate in which the therapeutic agent and/or the ligand is covalently bonded to the bridging agent(s) in an inappropriate manner may not maintain functional activity). For example, an ACVMP-L-HSA conjugate displayed hepatocyte targeting and antiviral activity (Fiume, L., (1989) *Naturwissenschaften* 76:74-76; U.S. Patent No. 4,725,672) whereas an ACVMP-polylysine-galactose conjugate was inactive (Fiume, L. et al. (1986) *FEBS Letters* 203:203-206)

As used herein, the term "bridging agent" is intended to include molecules which couple a therapeutic agent to an ASGR ligand. The bridging agent used in the conjugates of the invention can be a crosslinker, a polyfunctional carrier molecule or both a crosslinker and a polyfunctional carrier molecule. Accordingly, in one embodiment of the

invention, the conjugate has the general formula A-B-C-D, wherein A is the therapeutic agent, B is a crosslinker, C is a polyfunctional carrier molecule and D is asialoorosomucoid. In another embodiment, the conjugate has the general formula A-C-D, wherein A is the therapeutic agent, C is a polyfunctional carrier molecule and D is asialoorosomucoid. In yet another embodiment, the conjugate has the general formula A-B-D, wherein A is the therapeutic agent, B is a crosslinker and D is asialoorosomucoid.

The term "crosslinker" is intended to include molecules which can function as bridging molecules between two other molecules by way of having two reactive functional groups, one of which reacts to form a covalent bond with the first molecule and the other of which reacts to form a covalent bond with the second molecule, thereby effectively connecting the two molecules together. Preferably, the crosslinker has two reactive functional groups of different functional moieties. Examples of suitable functional groups include amino groups, carboxyl groups, sulfhydryl groups and hydroxy groups. When one functional group of the crosslinker is reacted with a molecule (e.g., a therapeutic agent), the other functional group can be, if necessary, prevented from reacting with that molecule by means of a protecting group which modifies the second functional group of the crosslinker so that it cannot react with the molecule. After the first reaction is completed, the protecting group can be removed, restoring the second functional group, and then the second functional group can be reacted with another molecule (e.g., an ASGR ligand such as asialoorosomucoid).

The term "polyfunctional carrier molecule" is intended to include molecules which can function as bridging molecules between two or more other molecules by way of having multiple (i.e., more than two) reactive functional groups which can form covalent bonds with the other molecules, thereby effectively connecting the other molecules together. In general, a polyfunctional carrier has a polymeric structure and, preferably, the multiple functional groups of the polyfunctional carrier are of the same functional moiety. Examples of suitable functional groups include amino groups, carboxyl groups and aldehyde groups. Because of the multiple reactive functional groups present on the polyfunctional carrier molecule, multiple molecules can be coupled to it (i.e., many molecules of a therapeutic agent and/or ligand can be coupled to a single molecule of carrier). Thus, the molar substitution ratio of conjugates containing a polyfunctional carrier molecule generally is increased relative to conjugates which do not contain a polyfunctional carrier molecule. Increasing the molar substitution ratio of a conjugate can provide a means by which to increase the therapeutic index (i.e., therapeutic activity) of a conjugate.

According to this invention, several different coupling strategies can be used to conjugate a therapeutic agent to asialoorosomucoid or other ligand for ASGR using

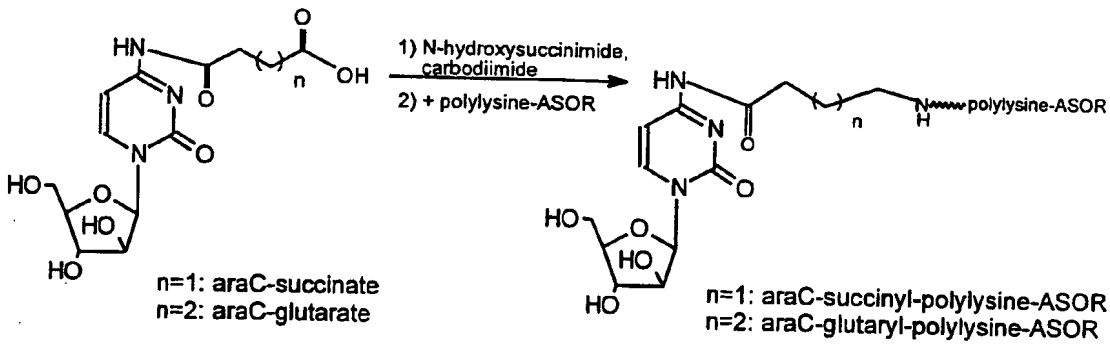
different crosslinkers and/or polyfunctional carrier molecules. The strategies and reactions are described in detail in the Examples. Different types of crosslinkers and polyfunctional carrier molecules which can be used are summarized briefly in the following subsections:

A. Acyl Crosslinkers

A therapeutic agent can be conjugated to a ligand (or a carrier-ligand complex) by preparing an acyl derivative of the agent, wherein the acyl derivative has a functional group which can react with another functional group on the ligand or on the carrier-ligand complex. The functional group of the acyl derivative can be, for example, a carboxyl group (which can then be reacted with amino groups on the ligand or carrier to form amide bonds), an amino group (which can then be reacted with carboxyl groups on the ligand or carrier to form amide bonds) or a phosphate group (which can then be reacted with amino groups on the ligand or carrier to form phosphoamide bonds).

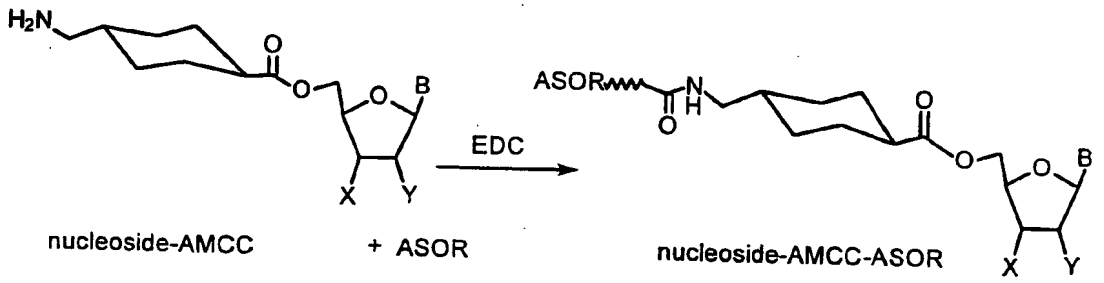
Carboxyacyl Crosslinkers:

A therapeutic agent can be conjugated to a ligand (or a carrier-ligand complex) by active ester coupling of a carboxyacyl derivative of the agent to a ligand or carrier-ligand having reactive amino groups. Briefly, a therapeutic agent is acylated at an amino or hydroxy group to form an acyl derivative. Preferred acyl derivatives are glutaryl and succinyl derivatives. Carboxyacyl derivatives of therapeutic agents (e.g., nucleoside analogs) can be prepared as previously described (see for example Erlanger, B.F., et al. (1967) *Methods Immun. Immunochem.* 1:144) and as detailed in Examples 1 and 2. The derivative carboxyacyl group of the therapeutic agent functions as a crosslinker to allow conjugation of the therapeutic agent to a ligand or carrier-ligand complex. The carboxyacyl derivative of the agent is activated (for example, with N-hydroxysuccinimide) to form an active ester. The actived carboxyacyl compound is then reacted with a ligand or a carrier (e.g., in a carrier-ligand complex, such as polylysine-ASOR) having functional amino groups to form amide bonds between the carboxyacyl crosslinker and the amino groups of the carrier or ligand, thereby coupling the agent to the carrier or ligand. The carboxyacyl derivative can be conjugated to a carrier or ligand as described in detail in Examples 1 and 2. A preferred polymeric carrier molecule to which carboxyacyl derivatives of therapeutic agents can be conjugated is a polyamino acid with reactive amino groups, such as polylysine or polyornithine. The polymeric carrier molecule can first be coupled to a ligand, such as ASOR, as described in Example 1 and then the carboxyacyl-derivative of the therapeutic agent can be conjugated to the carrier-ligand comples. For example, a carboxyacyl derivative of the nucleoside analog *ara*C can be conjugated to polylysine-ASOR as follows:



Aminoacyl Crosslinkers

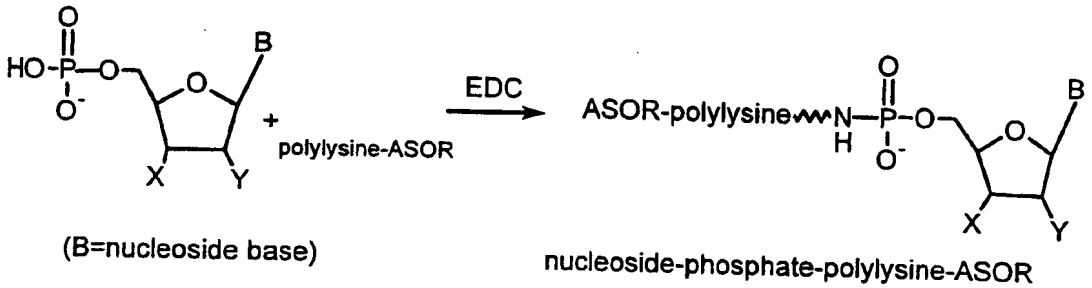
An amino derivative of a therapeutic agent can be conjugated to a ligand or carrier-ligand complex having reactive carboxyl groups through formation of amide bonds between the amino group of the derivatized therapeutic agent and the carboxyl groups of the ligand or carrier. The amino derivative of the therapeutic agent is conjugated to the ligand or carrier by carbodiimide coupling. A preferred amino derivative of a therapeutic agent is an aminoacyl derivative. For example, an aminomethylcyclohexanecarboxyl or 4-aminobutyryl derivative of the therapeutic agent can be prepared and coupled to a ligand or carrier-ligand as described in detail in Example 4. To prepare the aminoacyl derivative of the therapeutic agent, the amino group of an aminocarboxylic acid (e.g., aminomethylcyclohexanecarboxylic acid (AMCC) or aminobutanoic acid (GABA)) is protected, for example by Schotten-Bauman carbamoylation, and the protected aminocarboxylic acid is reacted with the therapeutic agent by carbodiimide-promoted esterification. Following this reaction, the protecting group is removed by hydrogenolysis (Brown , C.A. and Brown, H.C. (1966) *J. Org. Chem.* 31:3989-3995) and the aminoacyl derivative of the agent is coupled to a ligand or carrier-ligand complex having reactive carboxyl groups by carbodiimide coupling (e.g., with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride). For example, an aminomethylcyclohexanecarboxyl (AMCC)-derivative of a generic nucleoside analog can be conjugated to ASOR as follows:



The aminoacyl group can be coupled to the therapeutic agent through a reactive hydroxyl group (e.g., the 5' -OH of a nucleoside analog as shown above) or through a reactive amino group (e.g., the N^4 group of cytosine-derived nucleoside analogs such as araC and ddC). If the therapeutic agent has both a reactive hydroxyl group and a reactive amino group, one group can be protected during aminoacylation. For example, the 5' -OH of a cytosine nucleoside analog can be protected with a trityl group during aminoacylation and then detritylated (see Example 4).

B. Phosphate Crosslinkers

A phosphate derivative of a therapeutic agent can be conjugated to a ligand or carrier-ligand complex having reactive amino groups through formation of phosphoamide bonds between the phosphate group of the therapeutic agent and amino groups of the ligand or carrier. A therapeutic agent can be phosphorylated by standard procedures or a phosphate derivative of the agent can be obtained commercially. For example, 5' monophosphate derivatives of certain nucleoside analogs can be obtained commercially (e.g., araA-monophosphate; *ara*C-monophosphate). Procedures suitable for phosphorylating nucleoside analogs are described in Fiume, L., et al. (1989) *Naturewissenschaften* 76:74-76 and Sowa, T. and Ouchi, S. (1975) *Bulletin of the Chemical Society of Japan* 48(7):2084-2090 and in Example 3. The phosphate derivative of the therapeutic agent can then be conjugated to a ligand or carrier-ligand complex (e.g., polylysine-ASOR) which has reactive amino groups. For example, polyamino acids such as polylysine and polyornithine can be used as carrier molecules. The phosphate derivative of the therapeutic agent is coupled to the ligand or carrier-ligand complex by carbodiimide coupling (such as with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, EDC) to form phosphoamide bonds as described in detail in Example 3. For example, a 5' monophosphate derivative of a generic nucleoside analog can be conjugated to polylysine-ASOR (prepared as described in Example 1) as follows:



(B=nucleoside base)

nucleoside-phosphate-polylysine-ASOR

C. Peptide Crosslinkers

A preferred type of crosslinker for use in the conjugates is a peptide crosslinker which can be hydrolyzed intracellularly (e.g., by lysosomal enzymes) to release the therapeutic agent from the conjugate. Drug conjugates prepared with a peptide crosslinker have been found to be stable in serum *in vivo* and to release the drug in active form intracellularly through the action of lysosomal hydrolases (Trouet, A., et al., (1982) *Proc. Natl. Acad. Sci. USA* 79:626-629). A preferred peptide contains the amino acid sequence leucine-alanine-leucine (LAL) and is at least a tripeptide or a tetrapeptide. Structurally, a peptide has both a reactive amino group (i.e., the N-terminal end) and a reactive carboxy group (i.e, the C-terminal end). In a preferred embodiment, the peptide is used as a crosslinker between a therapeutic agent and a ligand (or carrier-ligand) in a C-terminal to N-terminal orientation (i.e., the C-terminal end of the peptide is coupled to the therapeutic agent and the N-terminal end is coupled to the ligand or carrier-ligand).

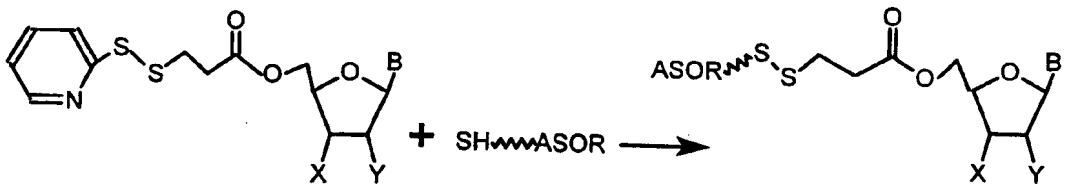
Accordingly, a peptide can be coupled to a therapeutic agent which has a reactive amino group by formation of an amide bond between the amino group of the therapeutic agent and the C-terminal carboxyl group of the peptide as described in detail in Example 5. For example, a peptide can be coupled to the the N^4 group of cytosine-derived nucleoside analogs such as *ara*C and ddC. Other reactive groups on the therapeutic agent and peptide (e.g., the N-terminal amino group) can be prevented from reacting by use of protecting groups. For example, the amino group of the peptide can be protected as a carbamate using standard techniques known in the art and reactive hydroxy groups of a nucleoside analog (e.g., 3' and 5' -OH groups) can be protected with *tert*butyldimethylsilyl groups. The peptide and therapeutic agent can be coupled by active ester coupling or carbodiimide coupling to create a peptide derivative of the therapeutic agent. The peptide derivative of the therapeutic agent can be conjugated to a ligand or carrier-ligand complex having reactive carboxyl groups through formation of amide bonds between the (deprotected) N-terminal amino group of the peptide and the carboxyl groups of the ligand or carrier. The peptide derivative of the therapeutic agent can be conjugated to the ligand or carrier with carboxyl groups by carbodiimide coupling as described in detail in Example 5. For example, a leucine-alanine-leucine (LAL) tripeptide derivative of a therapeutic agent can be conjugated to ASOR or to a carrier-ASOR complex, such as a polyglutamic acid-ASOR or polyaspartic acid-ASOR complex. Additionally, a peptide-derivative of a therapeutic agent can be conjugated to an aldehyde containing ligand or carrier-ligand complex (e.g. polyaldehyde dextran-ASOR) by reductive amination.

Alternatively, the peptide could be used as a crosslinker between a therapeutic agent and a ligand (or carrier-ligand) in an N-terminal to C-terminal orientation (i.e., the N-terminal end of the peptide is coupled to the therapeutic agent and the C-terminal end is coupled to the ligand or carrier-ligand), such as when the therapeutic agent has a reactive carboxy group and the ligand or carrier has reactive amino groups (e.g., polylysine). Additionally, a diamine peptide or a dicarboxylic acid peptide could be used with appropriate therapeutic agents, ligands and carriers.

In a preferred embodiment, the peptide contains the amino acid sequence Leu-Ala-Leu. Additional amino acid residues can be added to the N-terminal or C-terminal end of this tripeptide. For example, Leu-Ala-Leu-Lys could be used. The side chains of amino acids contained within the peptide can also be used for coupling purposes. For example, the amino group of the side chain of Lys contained within a peptide can be used for coupling to carboxy groups (e.g. on polyglutamic acid) or the carboxy group of the side chain of Glu contained within a peptide can be used for coupling to amino groups (e.g., on polylysine).

D. Reductively-Labile Crosslinkers

A reductively-labile crosslinker can be coupled to a therapeutic agent and then this complex can be coupled to a ligand for ASGR through sulfhydryl groups of amino acid side chains of the ligand to form disulfide bonds between the crosslinker and the ligand as described in detail in Example 6. For example, a 3-(2-pyridyldithio)propionyl (PDP) derivative of the therapeutic agent can be prepared through a reactive amino or hydroxy group present on the therapeutic agent (e.g. the N^4 amino or 5' hydroxy group of a nucleoside analog). The PDP derivative of the therapeutic agent is then coupled to an ASGR ligand or a derivative thereof which reacts with thiol groups. For example, the PDP derivative of a generic nucleoside analog can be coupled to ASOR as follows:



nucleoside-PDP nucleoside-DP-ASOR

A thiol-derivative of a therapeutic agent can also be coupled to a polyfunctional carrier molecule with reactive thiol groups. For example, thiolated derivates

of a polyamino acid (e.g., polylysine or polyaspartate) can be prepared by reacting the polyamino acid with SPDP to form a PDP-derivative of the polyamino acid. The PDP-derivative of the polyamino acid can then be coupled to the PDP-derivative of the therapeutic agent by reduction of one of the PDP-derivatives followed by a thiol exchange reaction. Alternatively, a thiolized polysaccharide, such as a thiolized dextran, can be used as a polyfunctional carrier molecule with a thiol-containing crosslinker.

E. Polyfunctional Carriers with Multiple Amino Groups

A polyfunctional carrier molecule with multiple amino groups can be conjugated to an ASGR ligand by carbodiimide coupling to form amide bonds between the amino groups of the carrier and carboxyl side chains or C-terminus of the ligand. Alternatively, the polyfunctional carrier molecule or ligand can be derivatized to allow coupling of the carrier and the ligand via functional groups other than the amino groups of the carrier and the carboxyl groups of the ligand. For example, a thiol derivative of the carrier can be made and coupled to the ligand by a thio-ether linkage. Alternatively, a hydrazide derivative can be used.

Preferred carrier molecules with multiple amino groups are polyamino acids such as polylysine and polyornithine. Preferably, the amino acids of the polymer are the naturally-occurring L amino acids (e.g., poly-L-amino acids such as poly-L-lysine or poly-L-ornithine). For example, poly-L-lysine can be conjugated to ASOR as described in Example 1 and in U. S. Patent Application Serial No. 08/043,008 by Findeis et al., incorporated herein by reference. A carrier-ligand complex can be reacted with a therapeutic agent or with a crosslinker-therapeutic agent complex which reacts with amino groups in order to conjugate the therapeutic agent to the ligand via the carrier. For example, a phosphate, glutarate or succinate derivative of a therapeutic agent can be conjugated to a carrier having multiple amino groups as described above and in Examples 1-3.

Preferably, the polyfunctional carrier molecule with multiple amino groups is a polymer, such as a poly amino acid with repeating amino acid residues. The carrying capacity of a polymeric carrier molecule (i.e., the number of molecules which can be coupled to the carrier) is a function of the number of reactive groups present on the molecule, which increases as the size of the polymer increases. Therefore, the carrying capacity of a carrier molecule can be increased by using a larger (i.e., greater molecular weight) carrier. For example, poly-L-lysine of about 4000 daltons can be used in conjugates of the invention, or for a greater carrying capacity, poly-L-lysine of 10,000 daltons can be used. Poly-L-lysine of up to about 60,000 daltons can be used in the conjugates. Thus, the molar substitution ratio

of a carrier-containing conjugate can be increased by increasing the size of the carrier (see Example 9, Table 1).

F. Polyfunctional Carriers with Multiple Carboxyl Groups

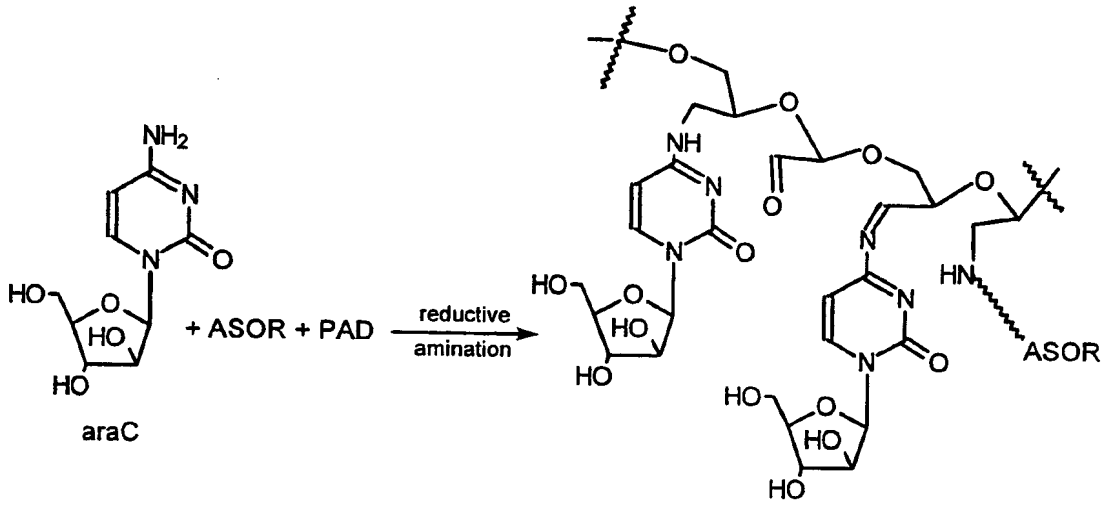
A polyfunctional carrier molecule with multiple carboxyl groups can also be used in the conjugate of the invention. Preferred carrier molecules with multiple carboxyl groups are polyamino acids such as polyglutamic acid and polyaspartic acid. Preferably, the polyamino acids are poly-L-amino acids, such as poly-L-glutamic acid or poly-L-aspartic acid. For example, a therapeutic agent with reactive amino groups (e.g., *ara*C) can be conjugated to a poly-L-glutamic acid carrier and the therapeutic agent-PLGA complex can be conjugated to ASOR as described in Example 7. A polyfunctional carrier molecule with multiple carboxy groups can be conjugated to an ASGR ligand by carbodiimide coupling to form amide bonds between the carboxyl groups of the carrier and amino side chains of the ligand. Alternatively, a crosslinker which reacts with carboxyl groups can be used as an intermediary between the therapeutic agent and the carrier with multiple reactive carboxy groups. For example, an aminoacyl or peptide derivative of a therapeutic agent can be conjugated to a carrier having multiple carboxyl groups.

As discussed above for carriers with multiple amino groups, the carrying capacity of a carrier molecule with multiple carboxyl groups can be increased by using a larger (i.e., greater molecular weight) carrier and thus the molar substitution ratio of a carrier-containing conjugate can be increased by increasing the size of the carrier. For example, poly-L-glutamic acid of about 14,000 daltons can be used in conjugate and a molar substitution ratio (drug:carrier) of 29 can be achieved with this size carrier (see Example 7). Poly-L-glutamic acid of up to about 60,000 daltons can be used in the conjugates.

G. Polyfunctional Carriers with Multiple Aldehyde Groups

Certain polymeric carrier molecules allow for conjugation of both the therapeutic agent and the ligand directly to the carrier molecule without the need for a crosslinker molecule as a bridging agent between the agent and the carrier. For example, a therapeutic agent with a reactive amino group can be conjugated to a carrier with multiple aldehyde residues by reductive amination. Additionally, a therapeutic agent with a hydrazone or hydrazide group can be coupled to a carrier with multiple aldehyde residues. A ligand can also be conjugated to the carrier through amino groups present on the ligand (e.g., amino groups of lysine side chains of the glycoprotein, such as ASOR) with polyaldehyde residues on the carrier by reductive amination. A preferred polymeric carrier molecule with polyaldehyde groups is a polysaccharide, such as polyaldehyde dextran. Polyaldehyde

dextran can be prepared from dextran by standard procedures (Bernstein, K, et al., (1978) *J. Natl. Cancer Inst.* 60(2):379-384; Foster, R.L. (1975) *Experientia*, 772-773) as described in Example 7. A therapeutic agent, such as a nucleoside analog, and a ligand, such as ASOR, can be then be conjugated to polyaldehyde dextran as described in Example 8. For example, araC and ASOR can be conjugated to polyaldehyde dextran as follows:



Alternative to directly conjugating the therapeutic agent to the carrier with multiple aldehyde groups, a crosslinker can be used as an intermediary between the therapeutic agent and the carrier. For example, a derivative of the therapeutic agent which provides a reactive amino group (i.e., an amino derivative such as an aminoacyl compound), a reactive hydrazine group or a reactive hydrazide group can be used to crosslink the therapeutic agent to a carrier with multiple reactive aldehyde groups.

IV. Coupling Strategies

The particular coupling strategy used to prepare a conjugate of the invention, that is, the particular crosslinker and/or polyfunctional carrier molecule used to conjugate a therapeutic agent to a ligand for ASGR (e.g., ASOR), will depend in part on the chemical structure of the therapeutic agent to be conjugated and thus can vary with different therapeutic agents. However, the coupling strategies used in the invention can be applied to a wide range of therapeutic agents. Therapeutic agents with a reactive amino, hydroxy, carboxyl, hydroxylamino, hydrazo or sulfhydryl group can be conjugated to a crosslinker or carrier molecule according to one or more of the coupling strategies described in the invention. When a therapeutic agent has multiple reactive groups, protecting agents can be

used (as described above and in the Examples) to direct a coupling reaction to a particular reactive group and then the protecting agent can be removed. When the ASGR ligand to be used in the conjugate is a glycoprotein, e.g., ASOR, the ligand possesses reactive amino groups and carboxyl groups, and possibly reactive sulfhydryl groups, from the side chains of amino acids and the N- and C-terminal ends of the glycoprotein. Thus, crosslinkers which react with any of these functional groups can be coupled to the ligand. Likewise, polyfunctional carrier molecules with either multiple amino groups, multiple carboxyl groups or multiple aldehyde groups can be coupled to the ligand (for example, ASOR can be conjugated to polylysine, polyglutamic acid or polyaldehyde dextran as described in the Examples). When both a crosslinker and a carrier molecule are used in the conjugate, an appropriate combination of crosslinker and carrier are chosen. For example, a crosslinker which reacts with amino groups is used with a carrier molecule having multiple amino groups. An appropriate combination can be selected from the groups of crosslinkers and carriers shown below:

Amino-Reactive Crosslinkers	Polyamino-Carriers
carboxyacyl (e.g., glutarate; succinate)	poly-L-lysine
phosphate	poly-L-ornithine

Alternatively, a crosslinker which reacts with carboxyl groups is used with a carrier molecule having multiple carboxyl groups. An appropriate combination can be selected from the groups of crosslinkers and carriers shown below:

Carboxyl-Reactive Crosslinkers	Polycarboxyl-Carriers
aminoacyl (e.g., AMCC, GABA)	poly-L-glutamic acid
peptide (e.g., Leu-Ala-Leu)	poly-L-aspartic acid

V. Activity of the Conjugates

The activity of the conjugates of the invention has essentially two components: the targeting activity of the ligand and the therapeutic activity of the therapeutic agent. The targeting activity of the ligand can be assessed *in vivo* by administering the conjugate intravenously into a subject (e.g., a mammal) and then measuring the rate of clearance of the conjugate from the circulation and/or measuring the association of the conjugate with target cells which express asialoglycoprotein receptors, for example liver cells. The clearance of the conjugate from the circulation and association of the conjugate with target cells can be compared relative to a non-conjugated therapeutic agent and relative to the association of the conjugate with other organs. A conjugate can be directly detected by

labeling it with a detectable substance, for example a radioactive isotope, to follow its distibution in a subject. For example, a therapeutic agent can be labeled with a radioactive isotope such as tritium or ^{14}C or the ligand can be labeled with ^{125}I.

Alternatively, the distribution of a conjugate can be assessed by its ability to competitively inhibit the binding of another asialoglycoprotein to ASGR (see for example Keenan-Rogers, V. and Wu, G.Y. (1990) *Cancer Chemother. Pharmacol.* 26:93-96). In this case, an unlabeled conjugate is coadministered with labeled asialoglycoprotein. For example, an unlabeled ASOR-containing conjugate can be coadministered with a labeled asialofetuin. The clearance of the labeled asialoglycoprotein from the circulation and/or the association of the labeled asialoglycoprotein with the liver is measured with and without coadministration of the conjugate. A conjugate which is effectively targeted to liver cells will decrease the rate of clearance of the labeled asialoglycoprotein from the circulation and decrease the association of the labeled asialoglycoprotein with the liver by competing with the labeled asialoglycoprotein for binding to asialoglycoprotein receptors on liver cells.

Additionally, the amount of a conjugate in the circulation can be assessed by immunological or chemical methods. For example, plasma samples can be collected at various times following intravenous injection of the conjugate and the amount of conjugate present therein can be determined by HPLC or by an immunological assay, such as a radioimmunassay or ELISA (for example, using an antibody against the ligand or carrier portion of the conjugate). Furthermore, the distribution of the conjugate can be assessed by autoradiographic or nuclear imaging methods using a radiolabeled conjugate (for instance a conjugate in which the ligand is labeled with ^{125}I).

The activity of the therapeutic agent in a conjugate can be assessed by measuring the therapeutic effectiveness of the conjugate against a disease or disorder to be treated by the therapeutic agent using an appropriate assay. For example, the antiviral activity of an anti-viral agent can be determined by measuring the amount of viral DNA replication or viral particle (or marker) production which occurs in the presence or absence of the conjugate relative to the unconjugated therapeutic agent. The effect of a conjugate on viral DNA replication can be assessed *in vitro* using a virally-infected cell line which expresses asialoglycoprotein receptors. For example, a hepatocyte cell line can be used. Hepatocyte cell lines have been transfected with hepatitis B virus DNA to create stable cell lines which transcribe HBV genes, translate HBV proteins and accumulate HBV DNA replicative intermediates. Such cell lines can be used to assess the anti-viral activity of conjugates. Appropriate HBV DNA-containing cell lines which can be used include the human hepatoblastoma (HepG2)-derived cell line, 2.2.15 (Sells, M.A., et al., (1987) *Proc. Natl. Acad. Sci. USA* 84:1005-1009; Sells, M.A., et al., (1988) *J. Virol.* 62:2336-2344) and

the human hepatoblastoma (Huh 6)-derived cell line HB 611 (Tsurimoto, T., et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:444-448).

Viral DNA-containing cells *in vitro* can be treated with various concentrations of a conjugate and the corresponding unconjugated therapeutic agent and the effect of the treatments on intracellular and/or extracellular viral DNA production can be determined. Intracellular DNA can be isolated from cells and extracellular DNA can be isolated from the culture medium. The DNA can then be analyzed by a hybridization procedure (e.g., dot blot hybridization, Southern blot etc.) or other appropriate DNA analysis procedure. For example, assays such as those described by Korba, B.E. and Gerin, J.L. ((1992) *Anti-viral Research* 19:55-70) and Ueda et al. ((1989)*Virology* 169:213-216) can be used. In the case of hepatitis B virus, the effect of the free and conjugated agent on different forms of HBV DNA can be measured. For example, the accumulation of relaxed circular DNA, replicative intermediates and integrated HBV DNA can be determined as described in Example 10. Since the amount of integrated (i.e, non-replicating) HBV DNA should not change upon treatment with either the free or conjugated agent, this DNA can be used as an internal control. The ID_{50} (i.e., dose necessary to inhibit 50 % of the viral DNA replication) can be determined for the conjugated and unconjugated agent to assess the therapeutic effectiveness of the conjugate. Additionally, production of viral antigens *in vitro* can be assessed to determine the therapeutic effectiveness of the conjugate.

The ability of the conjugates of the invention to target a therapeutic agent to a cell expressing asialoglycoprotein receptors can be assessed using cells in culture by comparing the cytotoxicity of the conjugates for $ASGR^+$ cells to the cytotoxicity of the conjugates for $ASGR^-$ cells. (This can then be compared to the cytotoxicity of the unconjugated therapeutic agent for $ASGR^+$ and $ASGR^-$ cells as a control). At a given dosage, a conjugate that is effectively targeted to $ASGR^+$ cells will be taken up to a greater extent by $ASGR^+$ cells than by $ASGR^-$ cells. Thus, a conjugate which effectively targets a therapeutic agent to $ASGR^+$ cells will be cytotoxic for $ASGR^+$ cells at a lower dosage than is needed to kill $ASGR^-$ cells. The cytoxicity of the conjugates of the invention can be measured using $ASGR^+$ and $ASGR^-$ cells as described in Example 10.

An appropriate animal model of a viral human disease can also be used to assess the anti-viral activity of anti-viral agent conjugates *in vivo*. For example, the effect of conjugated anti-viral agents can be assessed in *Ectromelia* virus-infected mice (for example, see Fiume, L., et al. (1981) FEBS Letters 129:261-264). Appropriate animal models exist for human hepatitis virus infection. For example, one animal model system for human hepatitis is woodchuck hepatitis virus (WHV)-infected woodchucks. Similar to humans, the Eastern woodchuck (*Marmota monax*) can be chronically infected with WHV. The genomic

organization of WHV is identical to HBV and the virological characteristics of the two diseases are similar (Summers, J., et al. (1975) *Proc. Natl. Acad. Sci. USA* 75:4533-4537; Galibert, F., et al. (1982) *J. Virol.* 41:51-65; Wong, D.C., et al., (1982) *J. Clin. Microbiol.* 15:484-490; Ponzetto, A., et al. (1984) *J. Virol.* 52:70-76; Ponzetto, A., et al. (1985) *Virus Res.* 2:301-315). Virally-infected animals can be injected intravenously with a conjugate or the corresponding free drug. Plasma levels of the conjugate can be measured as described above. The effect of the conjugated versus unconjugated agent on viral DNA replication can be determined, for example, by measuring serum levels of viral DNA. Other appropriate animal models for human hepatitis virus infection include duck hepatitis virus infection (Civitico, G., et al. (1990) *J. Med. Virol.* 31:90-97), ground squirrel hepatitis virus infection (Marion, P.L. et al. (1983) *Hepatology* 3:519-527) and, most preferably, infection of chimpanzees with human hepatitis virus (Thung, S.N. et al. (1981) Am. J. Pathology 105:328-332; Shouval, D, et al. (1980) *Proc. Natl. Acad. Sci. USA* 77:6147-6151).

The therapeutic activity of conjugates can also be assessed *in vivo* in human subjects. For example, humans chronically infected with HBV can be treated with a conjugate or the corresponding unconjugated agent. The effect of the conjugate on HBV infection can be determined by measuring the effect of the conjugate on one or more HBV markers, such as HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc or HBV DNA during the course of treatment.

VI. Uses of the Conjugates

The conjugates of the invention can be used to target a therapeutic agent to a cell of interest, i.e., a cell which expresses asialoglycoprotein receptors and to which delivery of the therapeutic agent is desired for therapeutic purposes. Asialoglycoprotein receptors are expressed on hepatocytes and thus a conjugate can target a therepeutic agent to hepatocytes. Galactosyl receptors have been reported to be present on rat testicular cells (Abdullah, M., et al. (1989) *J. Cell Biol.* 108:367-375) but these receptors are thought to differ structurally from hepatic ASGRs (i.e, be only partial receptors). Thus, the conjugates of the invention are not likely to be targeted to testicular cells. The conjugates of the invention therefore can be used to target a therapeutic agent selectively to hepatocytes. For example, conjugates comprising an anti-viral drug can be targeted to virally-infected hepatocytes. Alternatively, a cell can be engineered to express ASGR, for example by introducing into the cell a nucleic acid encoding the asialoglycoprotein receptor in a form suitable for expression of ASGR on the cell surface, to convert the cell into a target cell for the conjugates of the invention.

The conjugates can also be used to elicit a desired therapeutic effect in a subject. For example, a conjugate comprising an anti-viral drug can be used to treat a viral

infection, such as to decrease replication of viral DNA, inhibit viral particle replication and production, reduce symptoms of viral infection, etc. Because the conjugates are targeted to a tissue of interest and away from unaffected tissue (e.g., non-hepatic tissue), the non-specific toxicity of the therapeutic agent is diminished compared to the unconjugated agent. Additionally, because conjugation of a therapeutic agent to a targeting ligand results in increased delivery of the agent to the cell(s) of interest compared to unconjugated agent, the therapeutic index of the agent is increased, thereby providing therapeutically effective dosages at concentrations lower than is needed with the unconjugated agent.

The conjugates of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to target the therapeutic agent to cells expressing asialoglycoprotein receptors. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the conjugate to be administered in which any toxic effects are outweighed by the therapeutic effects of the conjugate. The term subject is intended to include living organisms in which a therapeutic agent can be targeted to cells expressing asialoglycoprotein receptors, e.g., mammals. Examples of subjects include humans, woodchucks, dogs, cats, mice, rats, and transgenic species thereof. Administration of a conjugate as described herein can be in any pharmacological form including a therapeutically active amount of conjugate alone or in combination with another therapeutic agent and a pharmaceutically acceptable carrier. For example, a conjugate of the invention can be coadministered with another therapeutic agent effective against a particular disease or condition to be treated. For example, a conjugate containing an anti-viral agent (e.g., a nucleoside analog) which is effective against hepatitis B virus can be administred together with an interferon, since interferons have also shown therapeutic activity against hepatitis B virus infection.

Administration of a therapeutically active amount of the conjugates of the invention is defined as an effective amount, at dosages and for periods of time, necessary to achieve the desired result. For example, a therapeutically active amount of a conjugate may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the conjugate to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (e.g., conjugate) is preferably administered intravenously (e.g., by injection). The active conjugate may be coated with or coadministered with a material to protect the conjugate from the action of enzymes, acids and other natural conditions which may inactivate the conjugate. For example, a conjugate may

be administered to an individual in an appropriate carrier or diluent and co-administered with enzyme inhibitors. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. The osmolarity of the compositions can be maintained in a physiological range by inclusion of appropriate amounts of compounds such as sugars, polyalcohols (e.g, mannitol or sorbitol) or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the conjugate in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., conjugate) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to

produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all patents, references and published patent applications cited throughout this application are hereby incorporated by reference.

The following general methodology was used in the Examples.

General Methods. Thin layer chromatography (TLC) was performed using glass backed Baker™ SI250F silica gel plates. Visualization was by ultraviolet irradiation or by dipping in an aqueous solution of 4.8 % ammonium molybdate, 0.2 % ceric ammonium nitrate, and 10 % sulfuric acid, followed by heating. Melting points were determined in capillary tubes using a Mel-Temp apparatus and are uncorrected. Dialysis was carried out in Spectrapor 12,000-14,000 MWCO tubing at 4 °C unless otherwise indicated. Ultraviolet/visible spectra were acquired on a Beckman DU70 spectrophotometer. Orosomucoid was purified from outdated plasma obtained from the American Red Cross Blood Bank. It was desialated at 80 °C, pH 1-2 for 60 min. Gel electrophoresis was carried out on a Novex Xcell II™ mini-gel system using 12 % SDS-PAGE denaturing nonreducing gels, unless otherwise indicated.

Polylysine, dextran, EDC, and the antiviral drugs *ara*A, *ara*C, ddC, acyclovir, *ara*AMP, *ara*CMP and AZT were obtained from Sigma. Other reagents were obtained from Aldrich.

The following abbreviations are used in the Examples and throughout the application:

ACV acyclovir
ACVMP acyclovir monophosphate
AMCC aminomethylcyclohexanecarboxylic acid
*ara*A adenosine arabinoside
*ara*AMP adenosine arabinoside monophosphate
*ara*C cytosine arabinoside
ASOR asialoorosomucoid
DCC dicyclohexylcarbodiimide
ddC dideoxycytidine
DMAP dimethylaminopyridine

DMF dimethylformamide
DMSO dimethylsulfoxide
EDC 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA ethylenediaminetetraacetic acid
FABMS fast atom bombardment mass spectrometry
HBV hepatitis B virus
MES 2-[*N*-Morpholino]ethanesulfonic acid
MSR molar substitution ratio
MWCO molecular weight cutoff
PAD polyaldehyde dextran
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PLL poly-L-lysine
PLGA poly-L-glutamic acid
PMS phenazine methosulfate
SDS sodium dodecyl sulfate
SPDP *N*-Succinimidyl 3-(2-pyridyldithio)propionate
TLC thin layer chromatography
Tris tris(hydroxymethyl)aminomethane
XTT 2,3-bis(2-methoxy-4-nitro-5-sulfophenol)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

EXAMPLE 1: Preparation of Drug Conjugates using a Glutarate Crosslinker and a Polylysine Carrier

In this example, asialoorosomucoid was prepared from orosomucoid and then conjugated to a polyfunctional carrier molecule with reactive amino groups, polylysine. Glutarate derivatives of different nucleoside analogs were prepared to provide a crosslinker which reacts with amino groups to enable conjugation of the nucleoside analogs to the polylysine-asialoorosomucoid complex. The glutarate derivatives of the nucleoside analogs were conjugated to the polylysine-asialoorosomucoid by active ester coupling. Glutarate derivatives of the nucleoside analogs *ara*C and *ara*A were prepared by modifications of previously described procedures and coupled to polylysine-asialoorosomucoid. Glutarate derivatives of the nucleoside analogs ddC, ACV and AZT were prepared as described herein and coupled to polylysine-asialoorosomucoid.

Orosomucoid (α-1-acid glycoprotein). Orosomucoid (OR) was isolated from human plasma. Human plasma was obtained from the American Red Cross Blood Services, Farmington, CT. Pooled human plasma (4 units, ~ 1.1 L) was transferred to dialysis tubing (12-14 Kd MWCO) and dialyzed overnight at 4 °C against 20 L of Buffer 1 (Buffer 1: 0.05M NaOAc, pH 4.5). The dialyzed plasma was then centrifuged at 10,000 rpm (15,000 x g) for 10 minutes at 4 °C. The supernatant was then filtered through Whatman #1 paper and the precipitate was discarded. the dialyzed and filtered plasma was applied to the DEAE-cellulose column. The column was prepared by suspending DEAE-cellulose (84g) in water, allowing it to swell for 2 h, and then washing successively with 0.5 N HCl, 0.5 N NaOH, and 0.01 M EDTA. The DEAE-cellulose was poured to a bed volume of 5 cm x 25 cm in a Waters AP-5 column. Using a peristaltic pump (10 mL/min flow rate) the column was equilibrated with Buffer 1 until the pH of the column eluate was 4.5. After the dialyzed and filtered plasma was applied to the column, the column was then washed with Buffer 1 until the eluate has an absorbance at 280 nm of less than 0.10. The column was then eluted with Buffer 2 (Buffer 2: 0.10 M NaAc, pH 4.0). The eluate was collected starting when the A_{280} began to increase and ending after the A_{280} had peaked and was < 0.10. After the orosomucoid-rich fraction had been eluted and collected, the column was washed with Buffer 3 (Buffer 3: 0.05 M NaAc, pH 3.0; 1L), and reequilibrated with Buffer 1.

The orosomucoid-rich eluate was brought to 50 % saturation with ammonium sulfate (313g/L of eluate) and stirred overnight at 4° C. This solution was then centrifuged (14,000 rpm x 15 min, 4° C) and the supernatant retained. Ammonium sulfate (320 g/L of 50 % saturation supernatant) was slowly added to bring the solution to 92 % saturation. This solution was then stirred for at least 4 h at 4 °C and then centrifuged (10,000 rpm x 30 min, 4 °C). The pellet was retained and dissolved in a minimal volume of water and transferred to dialysis tubing, leaving a 3-fold volume for expansion of the dialysate, and dialyzed for 2 days at 4 °C against 20 L of water (the water was changed after 1 day). The resulting dialysate was lyophilized and stored at -20 °C. The OR was run on SDS-PAGE and showed a single band at MW = 44 Kd (OR has a MW of 41,000 but runs on SDS-PAGE with an increased apparent MW) by staining with Coomasie blue. The typical yield of lyophilized salt-free OR using this procedure is 350-400 mg.

Asialoorosomucoid. Asialoorosomucoid was prepared from OR which was isolated as described above. OR was dissolved in water (10 mg/ml) and an equal volume of 0.1 N H_2SO_4 was added to the OR solution and the resulting mixture was heated at 80 °C for 1 h in a water bath to hydrolyze sialic acids from the protein. The acidolysis mixture was removed from the water bath, neutralized with NaOH, dialyzed against water for 2 days and then lyophilized. The thiobarbituric acid assay of Warren was then used to verify desialation of the OR (Sambrook, J., et al. (1989) Molecular Cloning, 2nd Ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor. Chapter 6). Targetability of ASOR samples was

verified by labeling with ^{125}I measuring liver uptake in rats/mice (Cristiano, R.J., et al. (1993) *Proc. Natl. Acad. Sci. USA*, 90:2122-2126.).

Poly-L-lysine-asialoorosomucoid conjugate (PLL-ASOR). The poly-L-lysine-asialoorosomucoid conjugate (PLL-ASOR) was prepared by carbodiimide coupling as follows. Asialoorosomucoid (200 mg, prepared as described above) and poly-L-lysine (160 mg) were dissolved in water (20 mL) and the pH adjusted to 7.5 with sodium hydroxide. EDC (94 mg) was added and the pH again adjusted to 7.5. The solution was stirred 16 h at 25 °C. Conjuages made using 4 Kd polylysine were then dialyzed sequentially against 4 L of 1 M guanidine, 4 L of 1 M sodium chloride, and 2 x 20 L of water, and lyophilized. Conjugates made using 10 Kd polylysine were purified by preparative acid-urea gel electrophoresis and then dialyzed against 4 L of 1 M sodium chloride, 20 L of water and lyophilized.

(N^4-(4-Carboxybutyryl)-1-β-D-arabinofuranosylcytosine (*ara*Cglutarate). *ara*Cglutarate was prepared according to Ishida, T., et al., U.S. Patent No. 3,991,045. Briefly, 300 mg of 1-β-D-arabinofuranosylcytosine was dissolved in 1.6 ml of water, and 5 ml of dioxane ws added, followed by further addition of 415 mg of glutaric anhydride. The mixture was stirred at room temperature for 48 hours. The reaction mixture was concentrated at reduced pressure at 60 °C to obtain a solid residue. The residue was dried in a vacuum desiccator to obtain a colorless transparent jelly-like substance. Chromotography on flash silica gel using ethyl acetate-methanol-acetic acid afforded the product (30 %). ^{1}H-NMR (CD_3OD) δ 1.76 (m, 2H), 2.10 (t, 2H), 2.35 (t, 2H), 4.05 (s, 2H), 4.28 (m, 3H), 5.91 (d, 1H), 6.08 (d, 1H), 7.56 (d, 1H).

*ara*Cglutarate-PLL-ASOR. *ara*Cglutarate (10 mg, 0.03 mmol; prepared as described above) was then dissolved in DMSO (0.25 mL). *N*-Hydroxysuccinimide (4 mg, 0.03 mmol) and EDC (7 mg, 0.036 mmol) were added and the solution was stirred for 16 h. The solution was then applied directly to a flash silica gel column (10 mm diameter) and eluted with chloroform:methanol (85:15). The partially pure *N*-hydroxysuccinimidyl ester (TLC, chloroform-methanol-acetic acid, 80:15:5, R_f = 0.24) thus obtained (20 μmol) was dissolved in DMSO (300 μL) and added dropwise to a solution of PLL-ASOR (10 mg in 400 μL, pH adusted to 7.5 with 1 N NaOH) at 4 °C with vigorous stirring. After 4 h the solution was applied to a Sephadex G25 column and eluted with PBS, pH = 6.8. The absorbance of the eluent was monitored at 260 nm, and the first peak to elute was dialyzed against water (2 x 2 L) and lyophilized. An aliquot of the lyophilized product was dissolved to 1 mg/mL in water and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 305, 285, 250 nm.

5'-O-(3-carboxybutyryl)-9-β-D-arabinofuranosyladenine (*araA*-glutarate). *araA*-glutarate was prepared according to Fiume, L. et al. (1980) *FEBS Letters* 116:185-188, using a slightly modified procedure. *ara*A (489 mg, 1.8 mmol) was dissolved in DMF (15 ml) with warming. Glutaric anhydride (261 mg, 2.3 mmol) and DMAP(22 mg, 0.2 mmol) were added and the solution was stirred for 18 h at 25 °C. The solvent was removed *in vacuo*, and the oil thus obtained was chromatographed on flash silica gel with chloroform-methanol-acetic acid (80:20:5) to afford 232 mg (33 %) of araAglutarate. Analysis: ^{1}H-NMR (DMSO-d_6) δ 1.41 (m, 2H), 1.92 (m, 2H), 2.04 (m, 2H), 2.11 (m, 2H), 2.52 (s, 1H), 3.70 (m, 2H), 3.87 (m, 1H), 4.49 (m, 1H), 5.13 (m, 1H) 5.22 (m, 1H), 5.28 (d, 1H), 6.45 (d, 1H), 7.29 (s, 1H), 8.13 (s, 1H), 8.26 (s, 1H); ^{13}C-NMR (DMSO-d_6) d 18, 30, 31, 59, 70, 76, 79, 81, 117, 138, 147, 151, 154, 170, 172.

*ara*A-glutarate-PLL-ASOR. *araA*-glutarate (20 mg, 0.05 mmol) was then dissolved in DMSO (0.7 mL). *N*-Hydroxysuccinimide (6 mg, 0.05 mmol) and EDC (31 mg, 0.16 mmol) were added and the solution was stirred 16 h. The solution was then applied directly to a flash silica gel column (10 mm diameter) and eluted with chloroform:methanol (80:20). A portion of the partially pure *N*-hydroxysuccinimidyl ester (TLC, chloroform-methanol-acetic acid, 80:20:5, R_f = 0.56) thus obtained (13 μmol) in DMSO (170 μL) was added dropwise to a solution of PLL-ASOR (10 mg in 378 μL, pH adusted to 7.5 with 1 N NaOH) at 4 °C with vigorous stirring. After 4 h the solution was applied to a Sephadex G25 column and eluted with PBS, pH = 6.8. The absorbance of the eluent was monitored at 260 nm. Fractions containing conjugate were pooled, dialyzed against water (2 x 2 L) and lyophilized. An aliquot of the lyophilized product was dissolved to 1 mg/mL in water and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 262 nm.

N^4-(4-Carboxybutyryl)-dideoxycytidine (ddCglutarate). ddCglutarate was prepared as follows. Glutaric anhydride (123 mg, 1.08 mmol) was added to a solution of 2',3'-dideoxycytidine (189 mg, 0.90 mmol) in DMF (8 mL), and the solution was stirred for 16 h at 25 °C. The solvent was removed *in vacuo*, affording a crystalline gum. Crystallization from 95 % ethanol afforded 157 mg of the product along with a small amount of impurity, as determined by TLC. Recrystallization gave 129 mg (44 %) of pure N^4-(4-carboxybutyryl)-2', 3'-dideoxycytidine. Analysis: TLC, R_f (chloroform-methanol-acetic acid, 80:20:5): 0.26; Melting point: 149 - 151 °C; ^{1}H-NMR (DMSO-*d6*/D_2O) δ 1.75 (t, J = 7.4 Hz, 2H), 2.23 (t, J = 7.4 Hz, 2H), 2.42 (t, J = 7.4 Hz, 2H), 3.58 (dd, J= 3.5 Hz, 12.1 Hz), 3.75 (dd, J = 3.0 Hz, 12.1 Hz), 4.11 (m, 1H), 5.92 (dd, J = 1.7 Hz, 6.5 Hz, 1H), 7.20 (d, J = 7.4 Hz, 1H), 8.47 (d, 7.5 Hz, 1H); IR: 3408, 2933, 1730, 1696, 1641, 1583, 1506, 1394, 1321, 1278, 1097, 821, 792 cm^{-1}; UV: λ_{max} 310, 273 nm.

ddCglutarate-PLL-ASOR. To a solution of N^4-(4-Carboxybutyryl)-dideoxycytidine (7 mg, 20 μmol), prepared as described above, in DMSO (180 μL) was added *N*-hydroxysuccinimide (3 mg, 0.03 mmol) and EDC (6 mg, 0.03 mmol). The solution was stirred for 2 h, then added directly to a solution of PLL-ASOR, prepared as described above, (28 mg in 400 μL water, pH adjusted to 7.5 with 0.1 N NaOH). The coupling reaction was allowed to proceed overnight at 4 °C, then diluted to 2 mL and dialyzed against 2 x 2 L of PBS, followed by 1 x 3 L of water. The product was lyophilized and analyzed by ultraviolet/visible absorption spectroscopy. λ_{max} 295, 245 nm.

9-(2-(4-Carboxybutyryloxy)-ethoxymethyl)guanine (ACVglutarate). Glutaric anhydride (196 mg, 1.70 mmol) and DMAP (13 mg, 0.11 mmol) were added to a suspension of 9-(2-hydroxyethyoxymethyl)guanine (189 mg, 0.90 mmol) in DMF (22 mL), and the suspension stirred for 16 h at 50 °C. An additional 10 mg (0.1 mmol) of DMAP was added and the mixture was heated to 65 °C, at which point a clear solution formed. The reaction was allowed to continue for 18 h, then the solvent was removed *in vacuo*, to afford a thick oil. The oil was suspended in hot ethanol, then chilled, filtered, and washed with cold ethanol yielding a white solid 300 mg (81 %). Analysis: TLC: R_f (chloroform-methanol-acetic acid, 80:20:5), 0.41; Melting point: 200 - 202 °C; ^{1}H-NMR (DMSO-d_6/D_2O) δ 1.69 (t, J = 7.3 Hz, 2H), 2.21 (t, J = 7.1 Hz, 2H), 2.29 (t, J = 7.5 Hz, 2H), 3.36 (bs, 2H), 4.08 (bs, 2H), 5.36 (s, 2H), 7.83 (s, 1H); IR (KBr): 3318, 3142, 2960, 2646, 1731, 1413, 1213, 1178, 1136, 1104, 752, 693 cm^{-1}; UV: λ_{max} 270, 255 nm.

ACVglutarate-PLL-ASOR. To a solution of 9-(2-(4-carboxybutyryloxy)-ethyoxymethyl)guanine (40 mg, 0.12 mmol), prepared as described above, in DMF (1.0 mL) was added *N*-hydroxysuccinimide (16.8 mg, 0.15 mmol) and DCC (35.6 mg, 0.17 mmol). The solution was stirred 18 h, filtered, and added to a solution of PLL-ASOR, prepared as described above, (10 mg in 208 μL water, pH adjusted to 7.5 with 0.1 N NaOH). The coupling reaction was allowed to proceed 3 h, then purified on a Sephadex G25 column with PBS, pH = 6.8. The absorbance of the eluent was monitored at 260 nm. Fractions containing conjugate were pooled and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 275, 245 nm.

5'-*O*-(4-Carboxybutyryloxy)-3'-azido-3'-deoxythymidine (AZTglutarate). A solution of 3'-azido-3'-deoxythymidine (100 mg, 0.37 mmol), glutaric anhydride (94 mg, 0.83 mmol), and DMAP (45 mg, 0.37 mmol) in DMF (3 mL) was stirred for 24 h at 25 °C. The solvent was removed *in vacuo* and the resulting oil chromatographed on a 20 mm column of flash silica gel with chloroform-methanol (9:1) as eluent. The product was isolated as an oil in 41 % yield. TLC: chloroform-methanol (8:2), R_f 0.56; ^{1}H-NMR (DMSO-d_6/D_2O) δ 1.73 (t, 2H), 1.80 (s, 3H), 2. 17 (t, 1H), 2.39 (m, 3H), 3.15 (s, 1H), 3.97 (dd, 1H), 4.25 (m, 2H), 4.45 (dd,

1H), 6.12 (ψt, 1H), 7.45 (s, 1H); IR: 3500, 3250, 2924, 2109, 1702, 1560, 1461, 1408, 1262, 1096, 801 cm^{-1}; UV: λ_{max} 267 nm.

AZTglutarate-PLL-ASOR. To a solution of AZTglutarate (11 mg, 30 μmol) in DMSO (250 μL), prepared as described above, was added *N*-hydroxysuccinimide (4 mg, 30 μmol) and EDC (7 mg, 36 μmol). The solution was stirred 18 h, then purified on a column of flash silica gel with chloroform-methanol (98:2) as eluent. The active ester thus obtained (R_f 0.35) was dissolved in 225 μL of DMSO and added dropwise to a solution of PL-ASOR (10 mg in 400 μL of water, pH adjusted to 7.5 with 0.1 N NaOH). The coupling reaction was allowed to proceed 4 h at 4 °C, then diluted and purified by dialysis against 6 L of PBS, pH = 6.8, followed by 1 x 20 L of water. The dialyzate was filtered through a 0.45 μ nylon membrane and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 267 nm.

EXAMPLE 2: Preparation of Drug Conjugates using a Succinate Crosslinker and a Polylysine Carrier

In this example, polylysine was conjugated to asialoorosomucoid as described in Example 1. Succinate derivatives of different nucleoside analogs were prepared to provide a crosslinker which reacts with amino groups to enable conjugation of the nucleoside analogs to the polylysine-asialoorosomucoid complex. The succinate derivatives of the nucleoside analogs were conjugated to the polylysine-asialoorosomucoid by active ester coupling. The succinate derivative of the nucleoside analog ACV was prepared be a modification of a previously described procedures and coupled to polylysine-asialoorosomucoid. The succinate derivative of the nucleoside analog *ara*A was prepared as described herein and coupled to polylysine-asialoorosomucoid.

9-(2-(3-carboxypropionyl)-ethyoxymethyl)guanine (ACVsuccinate). ACVsuccinate was prepared by a modification of the procedure described in Schaeffer et al. (1980) U.S. Patent No. 4, 199,574. Acyclovir (210 mg, 0.93 mmol), succinic anhydride (139 mg, 10.09 mmol) and DMAP (19 mg, 0.16 mmol) were combined in DMF (18 mL). The suspension was heated to 65 °C, at which point all components went into solution, and stirred for 16 h. The solvent was removed *in vacuo* and the resulting oil chromatographed on flash silica gel with chloroform-methanol-acetic acid (80:20:5) as eluent, affording ACVsuccinate (186 mg, 61 %). Analysis: TLC: R_f (chloroform-methanol-acetic acid, 80:20:5), 0.33; Melting point: 194-196 °C; ^{1}H-NMR (DMSO-d_6/D_2O) δ 2.50 (m, 4H), 3.66 (m, 2H), 4.09 (m, 2H), 5.35 (s, 2H), 7.82 (s, 1H); UV: λ_{max} 270, 252 nm.

ACVsuccinate-PLL-ASOR. To a solution of ACVsuccinate (37.5 mg, 0.12 mmol; prepared as described above) in DMF (1.0 mL) was added *N*-hydroxysuccinimide (16 mg, 0.15 mmol) and DCC (34 mg, 0.16 mmol). The solution was stirred 18 h, filtered, and added to a solution of PLL-ASOR (10 mg in 1 mL water, pH adjusted to 7.5 with 0.1 N NaOH). The coupling reaction was allowed to proceed 3 h, then purified on a Sephadex G25 column with PBS, pH = 6.8. The absorbance of the eluent was monitored at 260 nm. Fractions containing conjugate were pooled and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 277, 250 nm.

5'-*O*-(3-Carboxypropionyl)-9-β-D-arabinofuranosyladenine (*ara*Asuccinate). *ara*Asuccinate was prepared as follows. To a solution of 9-β-D-arabinofuranosyladenine (825 mg, 3.10 mmol) in DMF (25 mL) was added succinic anhydride (342 mg, 3.42 mmol) and DMAP (38 mg, 0.31 mmol). The reaction mixture was stirred 22 h, additional succinic anhydride (342 mg) and DMAP (38 mg) were added, and the reaction stirred 18 h. The solvent was removed *in vacuo* and the resulting oil chromatographed on flash silica gel using chloroform-methanol-acetic acid (80:20:5). The oily residue thus obtained was dissolved in methanol and precipitated in ether, affording an off-white, hygroscopic powder (320 mg, 28 %). Analysis: TLC: R_f (chloroform-methanol-acetic acid, 80:20:5), 0.46; MP = 145-147° C; IR(KBr): 2932(br), 1701, 1420, 1310, 1201, 921, 639 cm^{-1}; UV: λ_{max} 259 nm.

*ara*Asuccinate-PLL-ASOR. To a solution of 5'-*O*-(3-carboxypropionyl)-9-β-D-arabinofuranosyladenine (19 mg, 51 μmol), prepared as described above, in DMF (475 μL) was added *N*-hydroxysuccinimide (7.6 mg, 65 μmol) and DCC (16.2 mg, 78 μmol). The solution was stirred 18 h, filtered, and added to a solution of PLL-ASOR (5 mg in 475 μL water, pH adjusted to 7.5 with 0.1 N NaOH). The coupling reaction was allowed to proceed 3 h, then purified on a Sephadex G25 column with PBS, pH = 6.8. The absorbance of the eluent was monitored at 260 nm. Fractions containing conjugate were pooled and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 262 nm.

EXAMPLE 3: Preparation of Drug Conjugates using a Phosphate Crosslinker and a Polylysine Carrier

In this example, polylysine was conjugated to asialoorosomucoid as described in Example 1. Monophosphate derivatives of different nucleoside analogs were obtained or prepared to provide a crosslinker which reacts with amino groups to enable conjugation of the nucleoside analogs to the polylysine-asialoorosomucoid complex. The monophosphate derivatives of the nucleoside analogs were conjugated to the polylysine-asialoorosomucoid by carbodiimide coupling. The monophosphate derivatives of the nucleoside analogs *ara*C

and *ara*A were obtained commercially (Sigma). ACVmonophosphate was prepared as described below.

9-(2-hydroxyethyoxymethyl)guanine monophosphate (ACVMP). Phosphorus oxychloride (200 μL, 2.1 mmol), water (23 μL, 1.3 mmol) and pyridine (200 μL, 2.3 mmol) were combined in acetonitrile (0.5 mL) at 0-4 °C. Acyclovir (100 mg, 0.44 mmol) was added and the solution was stirred 4 h at 0-4 °C. The solution was then added to ice-water and stirred an additional hour. The pH was adjusted to 2 and the solution was applied to a prewashed column of charcoal-celite (2 g each). The column was further washed with 50 mL of water and the nucleoside products eluted with 50 mL of ethanol-water-ammonium hydroxide (10:9:1). This fraction was evaporated to dryness and redissolved in 200 mL of water. The pH was adjusted to 4 and the solution was applied to a 75 mL column of BioRad AG1-X8 resin, formate salt. The column was washed sequentially with 0.1 M, 1 M and 2 M formic acid. The 1M formic acid fractions contained pure ACVMP (65 mg; 48 %). ^{1}H-NMR (DMSO-d_6/D_2O) δ 3.66 (m, 2H), 3.90 (m, 2H), 5.39 (s, 2H), 8.10 (s, 1H).

*ara*Cmonophosphate-PLL-ASOR. 1-β-D-Arabinofuranosylcytosine monophosphate (3.3 mg, 10 μmol; obtained commercially from Sigma) and PLL-ASOR (10 mg) were dissolved in 168 μL water with pH adjustment to 7.5. The reaction was initiated at 4 °C by addition of EDC (2.7 mg, 14 μmol). After 2 h an additional 2.7 mg of EDC was added, and the reaction was left stirring at 4 °C 16 h. The solution was diluted to 2 mL, dialyzed against 1 x 3 L of 0.9 % NaCl followed by 1 x 3 L of water, lyophilized, and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 275 nm.

*ara*Amonophosphate-PL-ASOR. 9-β-D-Arabinofuranosyladenine monophosphate (4 mg, 10 μmol; obtained commercially from Sigma) and PLL-ASOR (10 mg) were dissolved in 520 μL of water with pH adjustment to 7.5. The reaction was initiated at 4 °C by addition of EDC (4 mg, 20 μmol). After 7 h the solution was diluted to 2 mL and dialyzed against 2 x 3 L of 0.9 % NaCl followed by 1 x 3 L of water. The product was lyophilized (14 mg), and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 260 nm.

ACV-monophosphate-PLL-ASOR. ACV-monophosphate (4 mg, 13 μmol), prepared as described above, and PLL-ASOR (22 mg) were dissolved in 600 μL of water with pH adjustment to 7.5. The reaction was initiated at 4 °C by addition of EDC (4.5 mg, 23 μmol). After 7 h the solution was diluted to 2 mL and dialyzed against 2 x 3 L of 0.9 % NaCl followed by 1 x 3 L of water. The product was lyophilized (18 mg), and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 260 nm.

EXAMPLE 4: Preparation of Drug Conjugates using an Aminoacyl Crosslinker

In this example, aminoacyl derivatives of nucleoside analogs were prepared to provide a crosslinker which reacts with carboxyl groups to enable conjugation of the nucleoside analogs to carboxyl groups on asialoorosomucoid. The aminoacyl crosslinkers used were derived from aminomethylcylcohexanecarboxylic acid (AMCC) and 4-aminobutanoic acid (GABA). The aminoacyl derivatives of the nucleoside analogs were conjugated to asialoorosomucoid by carbodiimide coupling.

5'-O-(trans-4-aminomethylcyclohexanecarboxyl)-9-β-D-arabinofuranosyl-adenine (*ara*A-AMCC). The benzyl carbamate of aminomethylcyclohexanecarboxylic acid was prepared by treatment of aminomethylcyclohexanecarboxylic acid (1 g, 6.4 mmol) dissolved in 5 mL of 2N NaOH with benzyloxycarbonyl chloride (1 mL, 7.0 mmol) slowly added with concurrent addition of an additional 1 mL of 2N NaOH at 5 °C. The mixture was stirred 1 h, then diluted with 25 mL water, and washed with 3 x 15 mL of ether. The aqueous solution was then acidified to a pH < 2.5. A voluminous white precipitate formed, which was filtered and washed with 500 mL of cold water and dried *in vacuo*. A portion of the product (27 mg, 0.1 mmol) was added to a solution of *ara*A (27 mg, 0.1 mmol) dissolved in DMF (0.6 mL). DCC (29 mg, 0.14 mmol) and DMAP (2.5 mg, 0.02 mmol) were added and the solution stirred 16 h. Purification by flash silica gel chromatography afforded Z-AMCC-*ara*A (13 mg, 25 %). Hydrogenolysis of Z-AMCC-*ara*A (185 mg, 0.34 mmol) afforded compound AMCC-*ara*A (117 mg, 0.28 mmol). Analysis: ^{1}H-NMR ($CDCl_3$) d 0.64 - 0.99 (m, 3H), 1.01 - 1.22 (m, 2H), 1.29 - 1.48 (m, 3H), 1.62 - 1.77 (m, 4H), 1.79 - 1.93 (s, 1H), 1.96 - 2.09 (m, 1H), 2.61 - 2.73 (d, 2H), 3.82 - 4.01 (m, 3H), 4.63 - 4.69 (yt, J = 6.6 Hz, 1H), 5.38 - 5.43 (ψt, J = 6.2, 1H), 6.51 - 6.57 (d, J = 6.1, 1H, H1'), 8.17 (s, 1H, H8), 8.31 (s, 1H, H2); UV: λ_{max} 260 nm.

*ara*A-AMCC-ASOR conjugate. *ara*A-AMCC (8 mg, 0.02 mmol), prepared as described above, dissolved in DMSO (60 μL) was added to ASOR (10 mg) in 200 μL of MES, 0.1M, pH = 5.6. EDC (16 mg, 0.08 mmol) was added and the solution was stirred 3.5 h at 4 °C. The solution was then chromatographed on Sephadex G25 with PBS as eluent. The first peak was collected and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 263 nm.

N^4-(4-Aminobutyryl)-1-β-D-arabinofuranosylcytosine (*ara*C-GABA). To a solution of 5'-*O*-trityl-1-β-D-arabinofuranosylcytosine (833 mg, 1.72 mmol) in DMF (12.5 mL) was added 4-(benzyloxycarbonylamino)-butanoic acid (1.38 g, 5.82 mmol) and DCC (365 mg, 1.76 mmol). The solution was stirred 5 h, then the solvent was removed *in vacuo* and the resulting oil chromatographed on a column of flash silica using chloroform-methanol (9:1) as eluent . A 238 mg aliquot of the product (R_f 0.38) thus obtained was detritylated by warming at

90 °C in 50 % acetic acid. Chromatography using the same eluent afforded pure *ara*C-GABA-Z (137 mg, 0.3 mmol). ^{1}H-NMR (CD_3OD) δ 1.85 (quin, 2H, $COCH_2CH_2CH_2NH$), 2.48 (t, 2H, $COCH_2CH_2CH_2NH$), 3.18 (t, 2H, $COCH_2CH_2CH_2NH$), 3.82 (m, 2H, H5 & 5'), 4.02 (m, 1H), 4.10 (ψt, J = 2.3 Hz, 1H), 4.25 (dd, J - 2.2 Hz, 3.5 Hz, 1H), 5.05 (s, 2H, benzylic), 6.19 (d, J = 3.7 Hz, 1H, H1'), 7.32 (m, 5H, phenyl), 7.42 (d, J = 7.5 Hz, 1H), 8.23 (d, J = 7.5, 1H). This compound was dissolved in 5 mL of ethanol:water: acetic acid (3:2:0.5) and hydrogenolyzed over 10 % Pd/C using the procedure of Brown (Brown, C.A. and Brown, H.C. (1966) *J. Org. Chem.* 31:3989-3995) until the starting material was no longer detectable by TLC and a non-migrating, ninhydrin positive spot appeared (about 30 min). The mixture was filtered through Celite, the ethanol removed at 20 - 30 mm Hg, and the resulting solution lyophilized to afford a white crystalline material. Melting point: 120 - 122 °C; IR (KBr): 3408(br), 2355, 1654, 1560, 1498, 1406, 1314, 1114, 1054, 804 cm^{-1}; UV: λ_{max} 305, 275 nm.

*ara*C-GABA-ASOR. *ara*C-GABA (10 mg, 30 μmol) in DMSO (100 μL) was added to a solution of ASOR (21 mg) in 420 μL of 0.1 M MES, pH 5.6. EDC (50 mg, 0.26 mmol) was added and the solution stirred for 3 h at 25 °C. The solution was chromatographed on a Sephadex G25 column with PBS, pH 6.5 as eluent. The first peak to elute was dialyzed against water and lyophilized.

EXAMPLE 5: Preparation of Drug Conjugates using a Peptide Crosslinker

In this example, a tripeptide derivative of a nucleoside analog was prepared to provide a crosslinker which reacts with carboxyl groups to enable conjugation of the nucleoside analog to carboxyl groups on asialoorosomucoid. The tripeptide derivative was conjugated to asialoorosomucoid by carbodiimide coupling.

3', 5'- Di-*O*-(*tert*butyldimethylsilyl)-1-β-D-arabinofuranosylcytosine (TBDMS-*ara*C). This compound was prepared according to Wipf, P. et al. (1991) *Bioorganic & Medicinal Chem. Letters* 1:745-750. A solution of 1-β-D-arabinofuranosylcytosine (1 g, 4.2 mmol), imidazole (2.2 g, 34 mmol), TBDMSCl (2.5 g, 17 mmol), and DMAP (53 mg, 0.4 mmol) was stirred 24 h at 25 °C. The mixture was then diluted with 50 mL of water and extracted with 3 x 50 mL of ether. The combined organic extracts were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Flash silica gel chromatography of the resulting oil afforded TBDMS-*ara*C. ^{1}H-NMR ($CDCl_3$) δ 0.11, 0.12, 0.13, 0.15 (4s, 12H, methyl), 0.90, 0.96 (2s, 18H, *t*butyl), 3.85 (m, 2H), 3.95 (m, 1H), 4.18 (m, 1H), 4.40 (m, 1H), 5.46 (d, J = 7.3 Hz, 1H, H1'), 6.14 (d, J = 5.1 Hz, 1H), 7.78 (d, J = 7.3 Hz, 1H).

N-(Benzyloxycarbonyl)-LeuAlaLeu (Z-LAL-OH). To a solution of *N*-(Benzyloxycarbonyl)-LeuAlaOH (2 g, 6 mmol), *N*-methylmorpholine (0.67 mL, 6 mmol) and *iso*butylchloroformate (0.82 mL, 6 mmol) in DMF (10 mL) was added a solution of Leu(O*t*Bu)·HCl (1.3 g, 6 mmol) and triethylamine (0.87 mL, 6 mmol) in THF (25 mL) at -5 °C. The mixture was allowed to come to room temperature, the solids removed by filtration, and washed with THF. Ethyl acetate was added, and the combined organic phases washed with 30 mL portions of water, saturated sodium bicarbonate, water, 1 % HCl and 2 x water. The organic phase was dried over magnesium sulfate, filtered and concentrated *in vacuo* to afford a white foam (1.5 g, 50 %). FABMS $[M + H]^+$ 506. To an aliquot of 300 mg (0.59 mmol) of this material in dichloromethane (3 mL) was add trifluoroacetic acid (1.5 mL). After 1.5 h the starting material was consumed as judged by TLC with chloroform-methanol (9:1). Removal of the solvents followed by filtration through a short column of flash silica gel with the same solvent afforded Z-LAL-OH (216 mg, 80 %). FABMS $[M + H]^+$ 450.

N^4-(LeuAlaLeu)-1-β-D-arabinofuranosylcytosine (*ara*C-LeuAlaLeu). To a solution of Z-LAL-OH (100 mg, 0.22 mmol; prepared as described above), *N*-methylmorpholine (25 μL, 0.22 mmol) and *iso*butylchloroformate (30 μL, 0.22 mmol) in THF (0.6 mL) was added a solution of TBDMS-*ara*C (79 mg, 0.22 mmol; prepared as described above) in THF (0.5 mL) at 0 °C. After 30 min stirring the solution was diluted with ether (15 mL) and extracted with 3 x 10 mL of water. The organic phase was dried over magnesium sulfate, filtered and concentrated *in vacuo*. The resulting oil was chromatographed on flash silica gel with 4 % methanol-chloroform to afford 80 mg (72 %) of TBDMS-*ara*C-(LeuAlaLeu-Z). A portion of this product (16 mg, 0.02 mmol) in THF (0.5 mL) was desilylated by treatment with tetrabutylammonium fluoride (10 mg, 0.04 mmol) for 1 h. Flash silica gel chromatography with 13 % methanol-chloroform afforded *ara*C-(LAL-Z) (10 mg, 74 %). ^{1}H-NMR (CD_3OD) characteristic signals: δ 0.91 (m, 12H, Leu CH_3), 3.80 - 4.55 (8H, arabinose & peptide α H's), 5.08 (2H, benzylic), 6.19 (1H, H1'), 7.30 (m, 4H, phenyl & 1 cytosine), 7.40 (1H, phenyl), 8.25 (1H, cytosine).

To remove the Z protecting group, this compound is dissolved in 5 mL of ethanol:water: acetic acid (3:2:0.5) and hydrogenolyzed over 10 % Pd/C using the procedure of Brown (Brown, C.A. and Brown, H.C. (1966) *J. Org. Chem.* 31:3989-3995) until the starting material is no longer detectable by TLC and a non-migrating, ninhydrin positive spot appears (about 30 min). The mixture is filtered through Celite, the ethanol removed at 20 - 30 mm Hg, and the resulting solution lyophilized.

araC-LeuAlaLeu-ASOR. To prepare araC-LeuAlaLeu-ASOR, TBDMS-*ara*C-LeuAlaLeu (30 μmol; prepared as described above) in DMSO (100 μL) is added to a solution of ASOR (21 mg) in 420 μL of 0.1 M MES, pH 5.6. EDC (50 mg, 0.26 mmol) is added and the

solution stirred for 3 h at 25 °C. The solution is chromatographed on a Sephadex G25 column with PBS, pH 6.5 as eluent. The first peak to elute is dialyzed against water and lyophilized.

EXAMPLE 6: Preparation of Drug Conjugates using a Reductively-Labile Crosslinker

In this example, a nucleoside analog was conjugated to asialoorosomucoid via a reductively-labile crosslinker.

N^4-(3-(2-Pyridyldithio)propionyl)-dideoxycytidine (ddC-PDP). Dideoxycytidine (40 mg, 0.2 mmol) and SPDP (60 mg, 0.2 mmol) in DMF (1 mL) were stirred 17 h at 25 °C. Purification on a 20 mm flash silica gel column yielded ddC-PDP (12 mg, 16 %). Analysis: ^{1}H-NMR (DMSO-d_6) characteristic signals: δ 5.91 (1H, H1'), 7.18, 8.49 (2H, cytosine), 7.25, 7.80, 8.47 (4H, pyridyl); UV: λ_{max} 295, 245; treatment with dithiothreitol resulted in the development of an absorbance maximum at 340 nm, characteristic of the liberation of pyridine-2-thione.

ddC-DP-ASOR. ASOR (4 mg), in 200 µL of degassed 50 mM borate, 1 mM EDTA, pH 8.5, was reacted for 1.5 h at 2 °C with 32 µL of a 9 mg/mL solution of 2-iminothiolane in the same buffer. The protein was separated from unreacted 2-iminothiolane using a PD10 column, eluted with degassed PBS, 1mM EDTA, pH 7.0. Fractions (0.5 mL) 6-8 were pooled and concentrated to 150 µL using a Centricon10™. An 80 µL aliquot was retained as a reference. To the remainder of the protein solution ddC-PDP (2 mg in 100 µL of DMF) was added dropwise with stirring at 4 °C. After 1.5 h at 4 °C the conjugate was separated from unreacted ddC-DP on a PD10 column as above. Analysis: PAGE, 45 % of the protein stained with coomassie blue migrated as a single band of M_r 36,000, 13 % with an M_r 80,000, and 14 % with an $M_r > 100,000$; UV: λ_{max} 280 nm.

EXAMPLE 7: Preparation of Drug Conjugates Using Polyglutamic Acid as a Carrier

In this example, a nucleoside analog was crosslinked to polyglutamic acid and then conjugated to ASOR by carbodiimide coupling.

*ara*C-PLGA. *ara*C was coupled to polyglutamic acid (PLGA; 14KD) according to the literature procedure (Kato, Y., et al. (1984) *Cancer Research* 44:25-30). Briefly, isobutyloxycarbonyl chloride (556 mg) and triethylamine (391 mg) were added to a solution of PLGA (500 mg) in dry DMF (40 ml) at -8 to -5 °C, and the mixture was stirred at this

temperature for 1 hour. To the resulting solution was added a solution of *ara*C (942 mg) in dry DMF (20 ml) and triethylamine (391 mg), and the reaction was allowed to proceed at 4 °C for three days and at room temperature for 4 hours. The reaction mixture was poured into cold 0.4 M phosphate buffer, pH 8.0 (20 ml), and any insoluble material was removed by filtration. The filtrate was dialyzed against 3 % NaCl solution and against water.

*ara*C-PLGA-ASOR. A 10 mg aliquot of araC-PLGA (prepared as described above) was combined with ASOR (15 mg) in water (0.5 mL). The pH was adjusted to 6.0, EDC (11 mg) was added, and the solution was stirred 16 h at 25 °C. The mixture was separated on a 2.5 x 100 cm Sephacryl S100 column with PBS, pH 6.7 as eluent at 0.3 mL/min.. Fractions of 8 mL were collected and fractions 28-32 were pooled and lyophilized (6 mg). Analysis: Non-reducing PAGE, M_r 46,000; UV: λ_{max} 295 nm, 248 nm; spectrophotometrically determined concentration of 97 μg *ara*C per mg of conjugate.

EXAMPLE 8: Preparation of Drug Conjugates using a Polyaldehyde Dextran Carrier

In this example, the polyfunctional carrier molecule polyaldehyde dextran (PAD) was prepared from dextran. Asialoorosomucoid was conjugated to PAD together with a cytosine-containing nucleoside analog, *ara*C or ddC, by reductive amination.

Polyaldehyde dextran (PAD). Polyaldehyde dextran was synthesized following literature precedence (Bernstein, K., et al. (1978) *J. Natl. Cancer Inst.* 60:379-384; Foster, R.L. (1975) *Experientia*, 772-773) using a 1:1 molar ratio of periodate:glucose monomer. Briefly, 1 g (0.108 mmol) of dextran (average molecular weight 9300) was dissolved in 186 mL of 0.03 M sodium periodate and stirred for 18 h at 25 °C in the absence of light. The solution was then dialyzed, in 3500 MWCO dialysis tubing, against 3 x 20 L followed by 1 x 5 L of water, and lyophilized.

*ara*C-PAD-ASOR. To PAD (16 mg in 300 μL of PBS, pH 7.2), prepared as described above, was added *ara*C (10 mg in 75 μL of PBS). The solution was stirred for 20 h at 25 °C after which time ASOR (6 mg in 200 μL of PBS) was added followed by an additional 20 h of reaction time. Sodium cyanoborohydride (6 mg, 90 μmol) was then added, and the solution stirred for 1.5 h at 37 °C. The conjugate was then separated from unreacted drug on a Sephadex G25 column with PBS as eluent. The first peak to elute was dialyzed against 4 L of water, lyophilized (16 mg), and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 278 nm.

ddC-PAD-ASOR. To PAD (15 mg in 275 µL of PBS, pH 7.2), prepared as described above, was added ddC (10 mg, 47 µmol). The solution was stirred 20 h at 25 °C after which time ASOR (6 mg in 200 µL of PBS) was added followed by an additional 20 h of reaction time. Sodium cyanoborohydride (6 mg, 90 µmol) was then added, and the solution stirred 1.5 h at 37 °C. The reaction mixture was then separated from unreacted drug on a Sephadex G25 column with PBS as eluent. The first peak to elute was dialyzed against 4 L of water, lyophilized (17 mg), and analyzed by ultraviolet/visible absorption spectroscopy: λ_{max} 277 nm.

EXAMPLE 9: Molar Substitution Ratios of Drug Conjugates

Determination of Molar Substitution Ratios. The concentration of drug on conjugates was determined spectrophotometrically using the equation:

$$D = \frac{A_{total} - (\varepsilon_{protein} \times C/d)}{\varepsilon_{drug} \times d \times 1000/C}$$

where D is the concentration of drug on the conjugate in µg/mL; ε are in $(mg/mL)^{-1}$; C is mg/mL of conjugate in the stock solution used; and d is the dilution of stock in the cuvette (1 cm path length).

The following assumptions were made: 1) the extinction coefficient of bound drug is the same as that of free drug; 2) the dry weight of the conjugate is the amount of protein and polymer (the weight of drug contributes less than 10 %); 3) the molecular weight of ASOR-polylysine conjugates is 40 Kd (amino acid analysis of sampled ASOR-polylysine conjugates supports a 1:1 ratio of ASOR (36 Kd):polylysine (4 Kd)). Extinction coefficients for the drugs used in the conjugates were determined by standard techniques.

The average molar substitution ratios for drug conjugates prepared in Examples 1-8 are shown in Table 1.

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Table 1

Average MSR[1] of Drug-Conjugates on ASOR.

Drug	Crosslinker	Carrier	MSR
*ara*C	glutarate	PLL[2]	6
	glutarate	PLL (10 Kd)	19
	phosphate	PLL	3
	———	PAD	37
	———	PLGA	16
	GABA	———	2
*ara*A	glutarate	PLL	5
	glutarate	PLL (10 Kd)	14
	succinate	PLL	5
	phosphate	PLL	7
	AMCC	———	4
ACV	glutarate	PLL	4
	succinate	PLL	5
	phosphate	PLL	1
ddC	glutarate	PLL	12
	glutarate	PLL (10 Kd)	17
	———	PAD	31
	DP	———	2
AZT	glutarate	PLL	16

[1]MSR expressed in terms of mol drug:mol ASOR.
[2]Poly-L-lysine (PLL) is 4 Kd unless otherwise indicated.

The molar substitution ratio for *ara*C-GABA-ASOR was determined by HPLC. HPLC was carried out on a Waters 600E solvent delivery system with a 486 absorbance detector and using an Applied Biosystems 4.6 mm x 100 mm C18 column. With a 25 min, 1 mL/min linear gradient of water to acetonitrile (each containing 0.1 % acetic acid), *ara*C eluted with a retention time of 12 min. Detection was at 260 nm. A Waters 743 Data module was used to quantitate peaks. Liberation of *ara*C by alkaline hydrolysis of the *N*-butyryl bond of the crosslinker was effected in 0.1 M, pH 9.5 borate buffer. A 1 mg/mL portion of the *ara*C-GABA-ASOR conjugate in this buffer was incubated at 37 °C. Injections of 20 µL aliquots were made initially, and after 1, 2, and 5 days. The amount of free *ara*C detected in the conjugate initially was < 3 µg/mg. After 24 h 20 µg of free drug had been released per mg of conjugate. A maximum of 25 µg/mg (corresponding to an MSR = 2) was detected after 5 days.

EXAMPLE 10: Inhibition of HBV DNA Replication by Drug Conjugates

The HBV-DNA transfected human hepatoblastoma (HepG2)-derived cell line, 2.2.15 (Sells, M.A., et al., (1987) *Proc. Natl. Acad. Sci. USA* 84:1005-1009; Sells, M.A. et al., (1988) *J. Virol.* 62:2336-2344), was used to evaluate the antiviral activity of the drug conjugates. Cells were either untreated, treated with a free drug, or treated with a conjugated drug (i.e., drug-PLL-ASOR). After exposure of 2.2.15 cells to the drug conjugate, the presence of HBV DNA extracellularly (i.e., DNA in virions released from the cells) intracellularly in the form of relaxed circular DNA, replication intermediates (single-stranded DNA and partial relaxed circles), and integrated HBV DNA was measured to determine the effect of the conjugate on viral DNA replication. The ID_{50}s (dose necessary to inhibit 50% of the viral DNA replication) for free and conjugated *ara*A, *acyclovir*, *ara*C, *AZT*, and *dd*C were determined relative to the untreated control. The CD_{50} (dose of drug required to kill 50% of the cells) was determined for free *ara*C and *dd*C and for one of the conjugates (ACVMP-PLL-ASOR), using both 2.2.15 cells and SKHep1 cells. In addition, to measure the level of clearance of the drug conjugates to the liver when administered *in vivo*, Balb/C mice were tail-vein injected with 10^6 cpm/μg of ^{125}I-radiolabeled drug conjugate and their livers excised and assayed for the presence of labeled drug conjugate after five minutes.

The results from the HBV antiviral assays (ID_{50}s) are summarized below in Table 2. Also shown in Table 2 are the results from the liver clearance assay (% to liver) and the CD_{50} measurements using both 2.2.15 (ASGR +) cells and SKHep 1 (ASGR -) cells. The experimental methods are also described in detail below.

Table 2

Drug Type	Crosslinker	% to Liver	HBV Inhibition extracellular HBV DNA (ID_{50})	HBV Inhibition intracellular and integrated HBV DNA (ID_{50})	CD_{50} 2.2.15 (ASGR +)	CD_{50} SKHep1 (ASGR -)
*ara*A	free drug		300μM			
	glutarate	97	15 μM			
	phosphate	96	30 μM			
acyclovir	free drug		>300 μM	>1 mM	>3 mM	>3 mM
	glutarate	98	30 μM			
	succinate	98	30 μM			
	phosphate	96	3 μM	23 μM	170 μM	>1 mM
	aminoacyl	98	70 μM			
*dd*C	free drug		60 μM	0.1 μM	3.5 mM	190 μM
	N-glutarate	99	>60 μM			
	PAD	98	1 μM	0.1 μM	3.5 μM	
AZT	free drug		> 3 mM			
	glutarate	99	150 μM			
*ara*C	free drug		0.4 μM	3.5 μM	3.5 μM	4.7 μM
	N-glutarate	96	6 μM	0.1-0.2 μM	2.4 μM	
	PAD		> 6 μM			

Experimental Methods

To assay HBV antiviral activity for drug conjugates containing *ara*A-glutarate-PLL-ASOR, *ara*A-phosphate-PLL-ASOR, *acyclovir*-glutarate-PLL-ASOR, *acyclovir*-succinate-PLL-ASOR, *acyclovir*-phosphate-PLL-ASOR, *acyclovir*-aminoacyl-PLL-ASOR, *dd*C-glutarate-PLL-ASOR, *dd*C-PAD-ASOR, *AZT*-glutarate-PLL-ASOR, *ara*C-glutarate-PLL-ASOR, and *ara*C-PAD-ASOR, stock cultures of 2.2.15 cells were maintained in RPMI 1640 supplemented with 5 % fetal bovine serum and 2 mM L-glutamine. The cells were incubated at 37 °C in a moist atmosphere containing 5 % CO_2. For antiviral treatment, 2.2.15 cells were seeded onto collagen-coated 24-well plates at a density of $4x10^4$ cells/cm^2. Confluent cultures (6-8 days post-seeding) were incubated in RPMI containing 2 % fetal bovine serum supplemented with increasing concentrations of either conjugate or free drug. The drug-containing medium was added on

day 1 (post-confluence) and replaced every 2 days (days 3, 5, 7 and 9) with medium containing fresh drug conjugate or free drug. On day 10, the medium and cells were collected for intracellular and extracellular HBV DNA analysis.

Ten days after drug treatment, the cells were lysed and total DNA was isolated. Total nucleic acids were extracted from conjugate-treated 2.2.15 cultures and intracellular HBV DNA was analyzed as follows. Cells were washed two times with excess Tris-Buffered Saline. The monolayer was lysed in 400 µl of lysis buffer (0.6 % SDS, 10 mM EDTA, 10 mM Tris-Cl pH 7.4) containing 20 µg/ml of RNase A and incubated at 37 °C for 30-60 minutes. The lysate was transferred to a microfuge tube, proteinase K was added to a final concentration of 100 µg/ml and incubated at 50 °C for at least 2 hours. The lysate was then adjusted to contain 300 mM sodium acetate, extracted once with phenol/chloroform/ isoamyl alcohol (25:24:1 v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). The DNA was concentrated by ethanol precipitation, resuspended in 50 µl of 10 mM Tris HCl, 1 mM EDTA, pH 8.0, and digested with the restriction enzyme Hind III. For Southern blotting, a third of the digested DNA was electrophoresed in a 0.8 % agarose gel and transferred to Micron Separations MagnaGraph® nylon membrane by overnight capillary transfer using 10X SSC transfer buffer. Hybridization was performed at 68 °C with a [^{32}P]dCTP-labeled EcoRI fragment of pADW-HTD (provided by T.J. Liang) containing the full length 3.2 genome of HBV. All labeling reactions were carried out with the Random Primers DNA Labeling System (BRL, Life Technologies). Levels of integrated DNA, relaxed circle DNA and replication intermediates were quantitated using a Packard Instant Imager and were graphed as a percentage of the untreated control.

The presence of HBV DNA intracellularly as relaxed circle, replication intermediate (single-stranded DNA and partial relaxed circle), and integrated HBV DNA was quantitatively compared. The relative amounts of relaxed circle DNA and replicative intermediates were normalized to the amounts of integrated DNA because the levels of integrated DNA should not be affected by antiviral treatment. The data for the *ara*C-glutarate-PL-ASOR conjugate in particular are plotted as a percent of the untreated control in Figure 1. The *ara*C-glutarate-PL-ASOR conjugate had a dose dependent inhibitory effect on intracellular HBV relaxed circle accumulation. The ID_{50} (dose necessary to inhibit 50 % of the viral DNA replication) for the relaxed circle DNA (final product) accumulation was at about 0.1 µM (30 ng/ml), whereas the replication intermediates continued to accumulate even at high concentrations (e.g., 0.5 mM (100 ng/ml)) of drug. The ID_{50} for free *ara*C was about 3.5 µM (750 ng/ml). However, this is the same value we determined to be the CD_{50} (dose of drug required to kill 50 % of the cells). Therefore, the increase of antiviral activity for the free *ara*C is probably due to cell death, rather than specific viral inhibition. Others have demonstrated that free *ara*C is not an inhibitor of HBV replication in 2.2.15 cells (B. Korba,

personal communication). Thus, by targeting *ara*C to cells via the asialoglycoprotein receptor, the antiviral activity of the drug may be enhanced.

Figure 2 shows the HBV antiviral activity of the *acyclovir*-phosphate-PLL-ASOR conjugate on intracellular HBV DNA, plotted as a percent of the untreated control. The conjugate inhibited at an ID_{50} of 23 μM (7 μg/ml), whereas free *acyclovir* had little effect on intracellular HBV DNA accumulation (ID_{50} of >1 Mm (>300 μg/ml)). These results demonstrate that *acyclovir* becomes a much more potent inhibitor of HBV replication when targeted to cells via the asialoglycoprotein receptor.

Figure 4 shows the effect of the ddC-PAD-ASOR conjugate on intracellular HBV DNA, plotted as a percent of the untreated control. Both the free *dd*C and the ddC-PAD-ASOR conjugate had an inhibitory effect on intracellular HBV relaxed circle and replication intermediate accumulation with an ID_{50} of about 0.1 μM (20 ng/ml).

The effect of the drug conjugates on extracellular HBV DNA (representing DNA in virions released from the cells) was also evaluated. For the analysis of extracellular DNA, the culture medium from the conjugate-treated 2.2.15 cells were centrifuged in a microfuge for 2 minutes to remove cellular debris. To denature the extracellular DNA, 400μl of the clarified medium was incubated for 20 minutes at room temperature (25 °C) in 1M NaOH.10X SSC (1X SSC is 0.15 M NaCl/0.015 M Sodium Citrate, pH 7.2). The samples were directly applied to nylon membranes (Micron Separations Systems MagnaGraph®) presoaked in 20X SSC using a slot blot apparatus (BRL). To neutralize the bound DNA, slots were washed twice with 0.5ml of 1M Tris, pH 7.2/2M NaCl and once with 0.5 ml of 20X SSC. The filters were removed, washed briefly in 2X SSC and UV crosslinked (Stratalinker, Strategene) prior to hybridization with the full length HBV probe (described above). The results are shown in Table 2 (above) and again demonstrate that by targeting drugs to hepatocytes via the asialoglycoprotein receptor, their antiviral activity against HBV can be significantly enhanced, as measured by extracellular HBV DNA accumulation.

In particular, as shown in Table 2, both conjugates of *ara*A (i.e., the glutarate conjugate and the phosphate conjugate) showed inhibition of HBV extracellular DNA at greater than ten fold lower concentrations than did free *ara*A. Likewise, all *acyclovir* conjugates had ID_{50}s far lower than free *acyclovir*. The most potent *acyclovir* conjugate, *acyclovir*-phosphate-PLL-ASOR, inhibited production of HBV extracellular DNA at a greater than one hundred fold lower concentration than did the free drug. The effect of the *acyclovir*-phosphate-PLL-ASOR conjugate on extracellular HBV DNA production is plotted as a percent of the untreated control in Figure 3. When the levels of extracellular DNA (representing DNA in virions released from the cells) were measured, it was found that the

conjugate inhibited at an ID_{50} of less than 3 μM (1 μg/ml), whereas free *acyclovir* had little effect on extracellular HBV DNA accumulation. For example, at 300 μM (100 μg/ml), the inhibition was only 40% (see Figure 3). Similarly, as shown in Table 2, the *dd*C-PAD-ASOR conjugate and the *AZT*-glutarate-ASOR conjugate both inhibited HBV more than one order of magnitude greater than did the free drugs. These results clearly demonstrate that by targeting antiviral agents to cells via the asialoglycoprotein receptor, their efficacy, as measured by extracellular HBV accumulation, is greatly increased compared to the free drugs.

The cytotoxicity of selected free and conjugated drugs were determined as follows. 2.2.15 and SK Hep1 cells were seeded on a 96 well microtiter plate at a density of $3.75x10^3$ cells/well. SK Hep1 cells do not have the receptor for ASOR and therefore serve as a control. Twenty four hours after seeding, increasing concentrations of free and conjugated drugs were added to the plates. Twenty four hours after drug addition, the drug was removed by a medium change. Seventy two hours after the initial drug application, the plates were stained with a combination of the tetrazolium reagent XTT which is metabolically reduced in viable cells to a water soluble formazan product and PMS which markedly enhances cellular reduction of XTT and allows direct absorbance readings. Staining was done according to Scudiero, D.A., et al., (1988) *Cancer Research*, 48:4827-4833. The absorbance was read at 450 nm. The percent survival was calculated by dividing the absorbance of each well by the 100% survival absorbance (no drug added) and multiplying by 100. The results are shown in Table 2. Less than 5-fold the amount of *acyclovir*-phosphate-PLL-ASOR was needed to kill the ASGR-expressing 2.2.15 cells than was needed to kill the ASGR negative SK Hep1 cells.

To measure the percent clearance of the drug conjugates to the liver, *in vivo* targeting assays were performed on mice as follows. Conjugates were iodinated using the chloramine-T procedure (Wood et al. (1981) *J. Clin. Chem. Clin. Biochem.* 19: 1051-1056). Balb/C mice were tail-vein injected with 10^6 cpm/μg of ^{125}I-drug conjugate in 0.5 ml PBS. The average specific activity was 10^6 cpm/μg. Animals were sacrificed by cervical dislocation at 5 minutes post injection. Five major organs (liver, spleen, kidneys, heart, and lungs) were excised and counted in the gamma counter to determine targeting of radiolabeled conjugate. As shown in Table 2, greater than 95% of all drug conjugates cleared to the liver, demonstrating that the drug conjugates are effectively delivered to liver cells *in vivo*.

EXAMPLE 11: Preparation of Drug Conjugates Containing Colchicine and a Reductively Labile Crosslinker

Drug conjugates of colchicine linked to ASOR were prepared as follows:

N-(3-(2-Pyridyldithio)propionyldeacetylcolchicine. SPDP (29 mg, 0.092 mmol) and DMAP (11 mg, 0.092 mmol) were added to deacetylcolchicine (33 mg, 0.092 mmol) in dichloromethane (1 mL), and the solution stirred for 2 hours at 23°C. The solution was then chromatographed on flash silica gel with 6% methanol in chloroform as a mobile phase. The first eluted colchicine derivative (21 mg, 0.038 mmol) was determined to be the correct product by ^{1}H-NMR ($CDCl_3$) δ 1.25 (s, 1H), 1.56 (s, 2H), 1.87 (m, 1H), 2.30 (m, 1H), 2.46 (m,1H), 2.53 (m, 1H), 2.67 (q, 2H), 3.03 (m, 2H), 3.65, 3.90, 3.94, 3.98 (4s, 12H), 4.68 (m, 1H), 6.53 (s, 1H), 6.80 (d, J = 10.8, 1H), 7.15 (m, 1H), 7.30 (m, 1H), 7.44 (s, 1H, H8), 7.56 (d, J = 8.2, 1H), 7.63 (m, 1H), 8.50 (d, J = 4.4 Hz, 1H).

Colchicine -DP-ASOR. 5 mg of SPDP in 0.06 mL of DMSO was added to 8 mg of ASOR in 0.5 mL of HEPES, 0.1 m, pH 7.5. The reaction mixture was stirred vigorously for 2 hours at 0 - 4°C. The mixture was then microfuged at 3000 rpm, 10 min. The supernatant was chromatographed on a PD10 column with sodium acetate, sodium chloride, 0.1 M pH 4.5 and the macromolecular fraction concentrated to 0.25 mL using a Centricon 10. Dithiothreitol (6 mg) in 0.25 mL of the same buffer was added and the solution stirred 30 min at 23°C. The solution was then chromatographed on a PD10 column with degassed PBS containing 1 mM EDTA and 0.02% sodium azide. To the macromolecular fraction was added N-(3-(2-pyridyldithio)propionyldeacetylcolchicine (2 mg) dissolved in 0.05 mL of DMSO. The mixture was stirred initially at 0-4°C, then at 23°C for 17 hours. N-Ethylmaleimide (1 mg) was added and the mixture stirred an additional 1 hour. The mixture was then microfuged at 5000 rpm, 10 min, and the supernatant chromatographed on a PD10 column in PBS. The macromolecular fraction was analyzed for colchicine by measuring absorbance at 353 nm, and for protein using the BioRad protein assay and PAGE. The conjugate contained 2 mol colchicine per mol ASOR.

The colchicine-DP-ASOR conjugates can be used in conjunction with other drug-containing conjugates of the invention (e.g., those described above in Examples 1-10), or with nucleic acid-containing conjugates, to increase delivery of the targeted drugs or nucleic acids to cells. It is believed that colchicine inhibits the translocation and/or fusion of endosomes to lysosomes. Therefore, when co-internalized into an endosome of a cell, along with other drug or nucleic acid-containing conjugates, the colchicine-DP-ASOR conjugate may prevent the degradation of the drug or nucleic acid-containing conjugates by lysosomes. Accordingly, in one embodiment of the invention, conjugates including colchicine or other

agents which inhibit the translocation and/or fusion of endosomes to lysosomes, and ASOR can be used to increase the antiviral activity or the level of expression of nucleic acids targeted to hepatocytes.

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EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A conjugate for targeting a therapeutic agent to a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-B-C-D, wherein:

A is a therapeutic agent;

B is a crosslinker which is covalently bonded to the therapeutic agent and to a polyfunctional carrier molecule;

C is a polyfunctional carrier molecule; and

D is a ligand for the asialoglycoprotein receptor selected from the group consisting of asialoorosomucoid, arabinogalactan and a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate, wherein the ligand is covalently bonded to the polyfunctional carrier molecule such that the ligand can bind to the asialoglycoprotein receptor.

2. The conjugate of claim 1 wherein the therapeutic agent is an antiviral agent.

3. The conjugate of claim 2 wherein the antiviral agent is effective against a hepatotropic virus.

4. The conjugate of claim 3 wherein the hepatotropic virus is selected from a group consisting of hepatitis A, hepatitis B, hepatitis C and hepatitis D.

5. The conjugate of claim 1 wherein the therapeutic agent is a nucleoside analog.

6. The conjugate of claim 5 wherein the nucleoside analog is selected from a group consisting of 9-β-D-arabinofuranosyladenine (araA), 9-β-D-arabinofuranosylcytosine (araC), dideoxycytidine (ddC), 9-(2-hydroxyethyoxymethyl)guanine (acyclovir; ACV) and 3'-azido-3'-deoxythymidine (AZT).

7. The conjugate of claim 1 wherein the crosslinker is covalently bonded to the polyfunctional carrier molecule through an amide bond.

8. The conjugate of claim 7 wherein the crosslinker is derived from a carboxyacyl compound.

9. The conjugate of claim 8 wherein the carboxyacyl compound is glutarate or succinate

10. The conjugate of claim 7 wherein the crosslinker is derived from an aminoacyl compound.

11. The conjugate of claim 10 wherein the aminoacyl compound is trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

12. The conjugate of claim 7 wherein the crosslinker is a peptide which is hydrolyzable intracellularly.

13. The conjugate of claim 12 wherein the peptide comprises an amino acid seqeunce Leu-Ala-Leu.

14. The conjugate of claim 1 wherein the crosslinker is covalently bonded to the polyfunctional carrier molecule through a phosphoamide bond.

15. The conjugate of claim 14 wherein the crosslinker is phosphate.

16. The conjugate of claim 1 wherein the polyfunctional carrier molecule is a poly-amino acid.

17. The conjugate of claim 16 wherein the poy-amino acid is polylysine or polyornithine.

18. The conjugate of claim 16 wherein the poly-amino acid polyglutamic acid or polyaspartic acid.

19. The conjugate of claim 1 wherein the polyfunctional carrier molecule is polyaldehyde dextran.

20. The conjugate of claim 1, wherein the cell is a hepatocyte.

21. A conjugate for targeting a therapeutic agent to a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-B-C-D, wherein:

A is a therapeutic agent;

B is a crosslinker which is covalently bonded to the therapeutic agent and to a polyfunctional carrier molecule;

C is a polyfunctional carrier molecule; and

D is asialoorosomucoid, wherein asialoorosomucoid is covalently bonded to the polyfunctional carrier molecule such that asialoorosomucoid can bind to the asialoglycoprotein receptor.

22. A conjugate for targeting a therapeutic agent to a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-C-D, wherein:

A is a therapeutic agent, which is covalently bonded to a polyfunctional carrier molecule;

C is a polyfunctional carrier molecule; and

D is a ligand for an asialoglycoprotein receptor, wherein the ligand is covalently bonded to the polyfunctional carrier molecule such that the ligand can bind to the asialoglycoprotein receptor.

23. The conjugate of claim 22 wherein the therapeutic agent is an antiviral agent.

24. The conjugate of claim 23 wherein the antiviral agent is effective against a hepatotropic virus.

25. The conjugate of claim 24 wherein the hepatotropic virus is selected from a group consisting of hepatitis A, hepatitis B, hepatitis C and hepatitis D.

26. The conjugate of claim 22 wherein the therapeutic agent is a nucleoside analog.

27. The conjugate of claim 26 wherein the nucleoside analog is selected from a group consisting of 9-β-D-arabinofuranosyladenine (araA), 9-β-D-arabinofuranosylcytosine (araC), dideoxycytidine (ddC), 9-(2-hydroxyethyoxymethyl)guanine (acyclovir; ACV) and 3'-azido-3'-deoxythymidine (AZT).

28. The conjugate of claim 22 wherein the polyfunctional carrier molecule has reactive aldehyde groups.

29. The conjugate of claim 28 wherein the polyfunctional carrier molecule is polyaldehyde dextran.

30. The conjugate of claim 22 wherein the polyfunctional carrier molecule is a poly-amino acid.

31. The conjugate of claim 22 wherein the ligand for the asialoglycoprotein receptor is asialoorosomucoid.

32. The conjugate of claim 22 wherein the ligand for the asialoglycoprotein receptor is arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate.

33. The conjugate of claim 22 wherein the cell is a hepatocyte.

34. A conjugate for targeting a therapeutic agent to a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-B-D, wherein:

A is a therapeutic agent selected from the group consisting of nucleoside analogs, reverse transcriptase inhibitors, topoisomerase inhibitors, DNA gyrase inhibitors and DNA binding agents;

B is a crosslinker which is covalently bonded to the therapeutic agent and asialoorosomucoid; and

D is asialoorosomucoid, wherein asialoorosomucoid is covalently bonded to the crosslinker such that asialoorosomucoid can bind to the asialoglycoprotein receptor.

35. The conjugate of claim 34 wherein the therapeutic agent is effective against a hepatotropic virus.

36. The conjugate of claim 35 wherein the hepatotropic virus is selected from a group consisting of hepatitis A, hepatitis B, hepatitis C and hepatitis D.

37. The conjugate of claim 34 wherein the nucleoside analog is selected from a group consisting of 9-β-D-arabinofuranosyladenine (araA), 9-β-D-arabinofuranosylcytidine (araC), dideoxycytidine (ddC), 9-(2-hydroxyethyoxymethyl)guanine (acyclovir; ACV) and

3'-azido-3'-deoxythymidine (AZT).

38. The conjugate of claim 34 wherein the crosslinker is covalently bonded to asialoorosomucoid through an amide bond.

39. The conjugate of claim 38 wherein the crosslinker is derived from an aminoacyl compound.

40. The conjugate of claim 39 wherein the aminoacyl compound is trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

41. The conjugate of claim 38 wherein the crosslinker is a peptide which is hydrolyzable intracellularly.

42. The conjugate of claim 41 wherein the peptide comprises an amino acid seqeunce Leu-Ala-Leu.

43. The conjugate of claim 34 wherein the crosslinker is covalently bonded to asialoorosomucoid through a disulfide bond.

44. The conjugate of claim 43 wherein the crosslinker is derived from (3-(2-pyridyldithio)propionate.

45. The conjugate of claim 34, wherein the cell is a hepatocyte.

46. A conjugate comprising 9-β-D-arabinofuranosylcytosine (*ara*C) and a ligand for an asialoglycoprotein receptor, wherein *ara*C is conjugated to the ligand by a crosslinker, a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule.

47. The conjugate of claim 46 wherein the ligand is asialoorosomucoid.

48. The conjugate of claim 46 wherein the ligand is arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate.

49. The conjugate of claim 47, wherein *ara*C is covalently bonded to a crosslinker selected from the group consisting of phosphate, glutarate and succinate, the crosslinker is covalently bonded to *ara*C and to a polyfunctional carrier molecule selected from the group consisting of polylysine and polyornithine, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

50. The conjugate of claim 47 wherein *ara*C and asialoorosomucoid are covalently bonded to polyaldehyde dextran.

51. The conjugate of claim 47, wherein *ara*C is covalently bonded to an aminoacyl crosslinker, the crosslinker is covalently bonded to *ara*C and to a polyfunctional carrier molecule selected from the group consisting of polyglutamic acid, polyaspartic acid and polyaldehyde dextran, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

52. The conjugate of claim 51 wherein the aminoacyl crosslinker is derived from trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

53. The conjugate of claim 51 wherein the aminoacyl crosslinker is a peptide comprising an amino acid sequence Leu-Ala-Leu.

54. A pharmaceutical composition comprising a solution of the conjugate of claim 47 and a physiologically acceptable carrier.

55. A conjugate comprising 9-(2-hydroxyethyoxymethyl)guanine (acyclovir; ACV) and a ligand for an asialoglycoprotein receptor wherein ACV is conjugated to the ligand by a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule.

56. The conjugate of claim 55 wherein the ligand is asialoorosomucoid.

57. The conjugate of claim 55 wherein the ligand is arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate.

58. The conjugate of claim 56, wherein ACV is covalently bonded to a crosslinker selected from the group consisting of phosphate, glutarate and succinate, the crosslinker is covalently bonded to ACV and to a polyfunctional carrier molecule selected from the group consisting of polylysine and polyornithine, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

59. The conjugate of claim 56, wherein ACV is covalently bonded to an aminoacyl crosslinker, the crosslinker is covalently bonded to ACV and to a polyfunctional carrier molecule selected from the group consisting of polyglutamic acid, polyaspartic acid

and polyaldehyde dextran, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

60. The conjugate of claim 59 wherein the aminoacyl crosslinker is derived from trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

61. The conjugate of claim 59 wherein the aminoacyl crosslinker is a peptide comprising an amino acid sequence Leu-Ala-Leu.

62. A pharmaceutical composition comprising a solution of the conjugate of claim 56 and a physiologically acceptable carrier.

63. A conjugate comprising dideoxycytidine (ddC) and a ligand for an asialoglycoprotein receptor wherein ddC is conjugated to the ligand by a crosslinker, a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule.

64. The conjugate of claim 63 wherein the ligand is asialoorosomucoid.

65. The conjugate of claim 63 wherein the ligand is arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate.

66. The conjugate of claim 64, wherein ddC is covalently bonded to a crosslinker selected from the group consisting of phosphate, glutarate and succinate, the crosslinker is covalently bonded to ddC and to a poly unctional carrier molecule selected from the group consisting of polylysine and polyornithine, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

67. The conjugate of claim 64 wherein ddC and asialoorosomucoid are covalently bonded to polyaldehyde dextran.

68. The conjugate of claim 64, wherein ddC is covalently bonded to an aminoacyl crosslinker, the crosslinker is covalently bonded to ddC and to a polyfunctional carrier molecule selected from the group consisting of polyglutamic acid, polyaspartic acid and polyaldehyde dextran, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

69. The conjugate of claim 68 wherein the aminoacyl crosslinker is derived from trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

70. The conjugate of claim 68 wherein the aminoacyl crosslinker is a peptide comprising an amino acid sequence Leu-Ala-Leu.

71. The conjugate of claim 64 wherein the crosslinker is derived from (3-(2-pyridyldithio)propionate.

72. A pharmaceutical composition comprising a solution of the conjugate of claim 64 and a physiologically acceptable carrier.

73. A conjugate comprising 9-β-D-arabinofuranosyladenine (*ara*A) and a ligand for an asialoglycoprotein receptor selected from the group consisting of asialoorosomucoid, arabinogalactan and a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate, wherein *ara*A is conjugated to the ligand by a crosslinker, a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule.

74. The conjugate of claim 73 wherein the ligand is asialoorosomucoid.

75. The conjugate of claim 74 wherein *ara*A is covalently bonded to a crosslinker selected from the group consisting of phosphate, glutarate and succinate, the crosslinker is covalently bonded to *ara*A and to a polyfunctional carrier molecule selected from the group consisting of polylysine and polyornithine, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

76. The conjugate of claim 74 wherein *ara*A is is covalently bonded to an aminoacyl crosslinker, the crosslinker is covalently bonded to *ara*A and to a polyfunctional carrier molecule selected from the group consisting of polyglutamic acid, polyaspartic acid and polyaldehyde dextran, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

77. The conjugate of claim 76 wherein the aminoacyl crosslinker is derived from trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

78. The conjugate of claim 76 wherein the aminoacyl crosslinker is a peptide comprising an amino acid sequence Leu-Ala-Leu.

79. A pharmaceutical composition comprising a solution of the conjugate of claim 74 and a physiologically acceptable carrier.

80. A conjugate comprising 3'-azido-3'-deoxythymidine (AZT) and a ligand for an asialoglycoprotein receptor wherein AZT is conjugated to the ligand by a crosslinker, a polyfunctional carrier molecule or a crosslinker and a polyfunctional carrier molecule.

81. The conjugate of claim 80 wherein the ligand is asialoorosomucoid.

82. The conjugate of claim 80 wherein the ligand is arabinogalactan or a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate.

83. The conjugate of claim 81, wherein AZT is covalently bonded to a crosslinker selected from the group consisting of phosphate, glutarate and succinate, the crosslinker is covalently bonded to AZT and to a polyfunctional carrier molecule selected from the group consisting of polylysine and polyornithine, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

84. The conjugate of claim 81, wherein AZT is covalently bonded to an aminoacyl crosslinker, the crosslinker is covalently bonded to AZT and to a polyfunctional carrier molecule selected from the group consisting of polyglutamic acid, polyaspartic acid and polyaldehyde dextran, and the polyfunctional carrier molecule is covalently bonded to asialoorosomucoid.

85. The conjugate of claim 84 wherein the aminoacyl crosslinker is derived from trans-4-aminomethylcyclohexanecarboxylate or 4-aminobutyrate.

86. The conjugate of claim 84 wherein the aminoacyl crosslinker is a peptide comprising an amino acid sequence Leu-Ala-Leu.

87. A pharmaceutical composition comprising a solution of the conjugate of claim 81 and a physiologically acceptable carrier.

88. A method for targeting a therapeutic agent to a cell with asialoglycoprotein receptors in a subject, comprising:

(a) forming a conjugate comprising a general formula A-B-C-D, wherein:

A is a therapeutic agent,

B is a crosslinker which is covalently bonded to the therapeutic agent and to a polyfunctional carrier molecule,

C is a polyfunctional carrier molecule,

D is a ligand for the asialoglycoprotein receptor selected from the group consisting of asialoorosomucoid, arabinogalactan and a Tris-(N-acetyl galactosamine aminohexyl glycoside) amide of tyrosyl(glutamyl)glutamate, wherein the ligand is covalently bonded to the polyfunctional carrier molecule such that the ligand can bind to the asialoglycoprotein receptor; and

(b) administering the conjugate in a physiologically acceptable carrier to the subject.

89. A method for targeting a therapeutic agent to a cell with asialoglycoprotein receptors in a subject, comprising:

(a) forming a conjugate comprising a general formula A-C-D, wherein:

A is a therapeutic agent, which is covalently bonded to a polyfunctional carrier molecule,

C is a polyfunctional carrier molecule,

D is a ligand for an asialoglycoprotein receptor, wherein the ligand is covalently bonded to the polyfunctional carrier molecule such that the ligand can bind to the asialoglycoprotein receptor; and

(b) administering the conjugate in a physiologically acceptable carrier to the subject.

90. A method for targeting a therapeutic agent to a cell with asialoglycoprotein receptors in a subject, comprising:

(a) forming a conjugate comprising a general formula A-B-D, wherein:

A is a therapeutic agent selected from the group consisting of nucleoside analogs, reverse transcriptase inhibitors, topoisomerase inhibitors, DNA gyrase inhibitors and DNA binding agents,

B is a crosslinker which is covalently bonded to the therapeutic agent and to asialoorosomucoid,

D is asialoorosomucoid, wherein asialoorosomucoid is covalently bonded to the crosslinker such that asialoorosomucoid can bind to the asialoglycoprotein receptor; and

(b) administering the conjugate in a physiologically acceptable carrier to the subject.

91. A method for targeting 9-β-D-arabinofuranosylcytosine (*ara*C) to a cell with asialoglycoprotein receptors in a subject comprising administering to the subject the pharmaceutical composition of claim 54.

92. A method for targeting 9-(2-hydroxyethyoxymethyl)guanine (ACV) to a cell with asialoglycoprotein receptors in a subject comprising administering to the subject the pharmaceutical composition of claim 62.

93. A method for targeting dideoxycytidine (ddC) to a cell with asialoglycoprotein receptors in a subject comprising administering to the subject the pharmaceutical composition of claim 72.

94. A method for targeting 9-β-D-arabinofuranosyladenine (*ara*A) to a cell with asialoglycoprotein receptors in a subject comprising administering to the subject the pharmaceutical composition of claim 79.

95. A method for targeting 3'-azido-3'-deoxythymidine (AZT) to a cell with asialoglycoprotein receptors in a subject comprising administering to the subject the pharmaceutical composition of claim 87.

96. A conjugate for targeting an agent that inhibits the translocation and/or fusion of endosomes to lysosomes in a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-B-D, wherein:

A is an agent that inhibits the translocation and/or fusion of endosomes to lysosomes;

B is a reductively labile crosslinker which is covalently bonded to the agent that inhibits the translocation and/or fusion of endosomes to lysosomes; and

D is a ligand for the asialoglycoprotein receptor, wherein the ligand is covalently bonded to the crosslinker such that the ligand can bind to the asialoglycoprotein receptor.

97. The conjugate of claim 96, wherein the agent that inhibits the translocation and/or fusion of endosomes to lysosomes is colchicine.

98. The conjugate of claim 97, wherein the reductively labile crosslinker is dithiopropionyl.

99. The conjugate of claim 96, wherein the ligand for the asialoglycoprotein receptor is an asialoglycoprotein.

100. The conjugate of claim 99, wherein the asialoglycoprotein is asialoorosomucoid.

101. A conjugate for targeting colchicine to a cell expressing an asialoglycoprotein receptor, the conjugate comprising a general formula A-B-D, wherein:

A is colchicine;

B is a reductively labile crosslinker which is covalently bonded to the colchicine; and

D is a ligand for the asialoglycoprotein receptor, wherein the ligand is covalently bonded to the crosslinker such that the ligand can bind to the asialoglycoprotein receptor.

102. The conjugate of claim 101, wherein the reductively labile crosslinker is dithiopropionyl.

103. The conjugate of claim 101, wherein the ligand for the asialoglycoprotein receptor is an asialoglycoprotein.

104. The conjugate of claim 103, wherein the asialoglycoprotein is asialoorosomucoid.

5

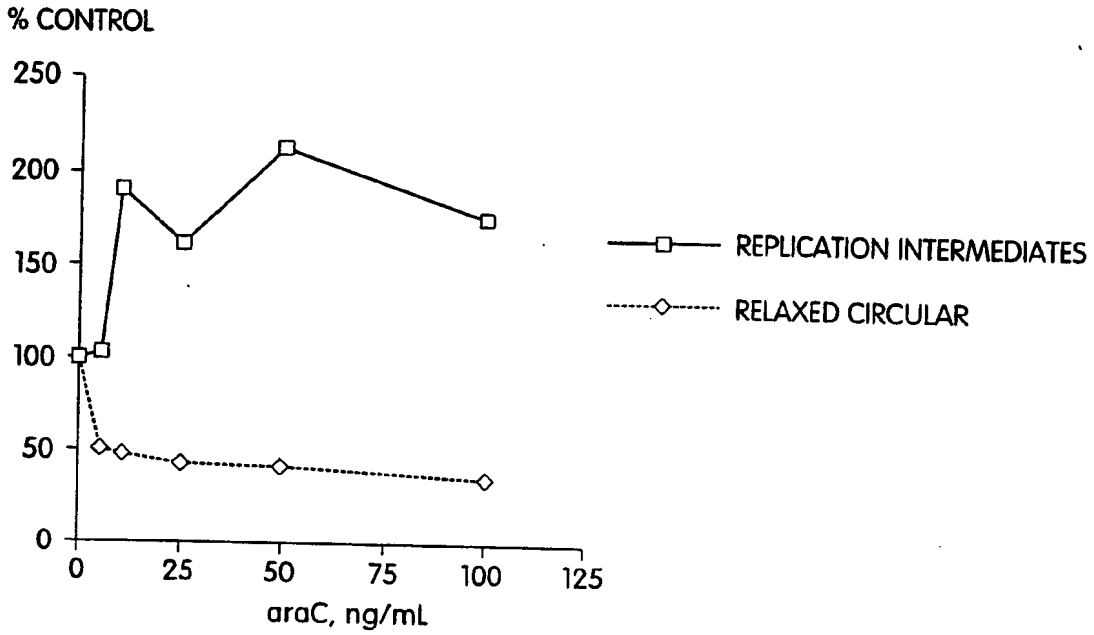
% CONTROL
250
200
150
100
50
0
0
25
50
75
100
125
araC, ng/mL
REPLICATION INTERMEDIATES
RELAXED CIRCULAR

Fig. 1

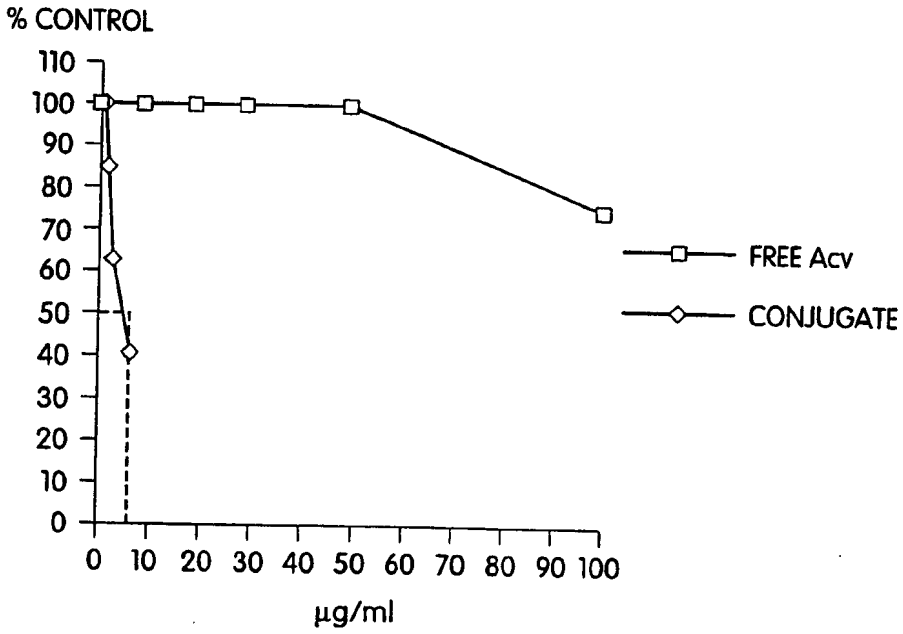
% CONTROL
110
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
µg/ml
FREE Acv
CONJUGATE

Fig. 2

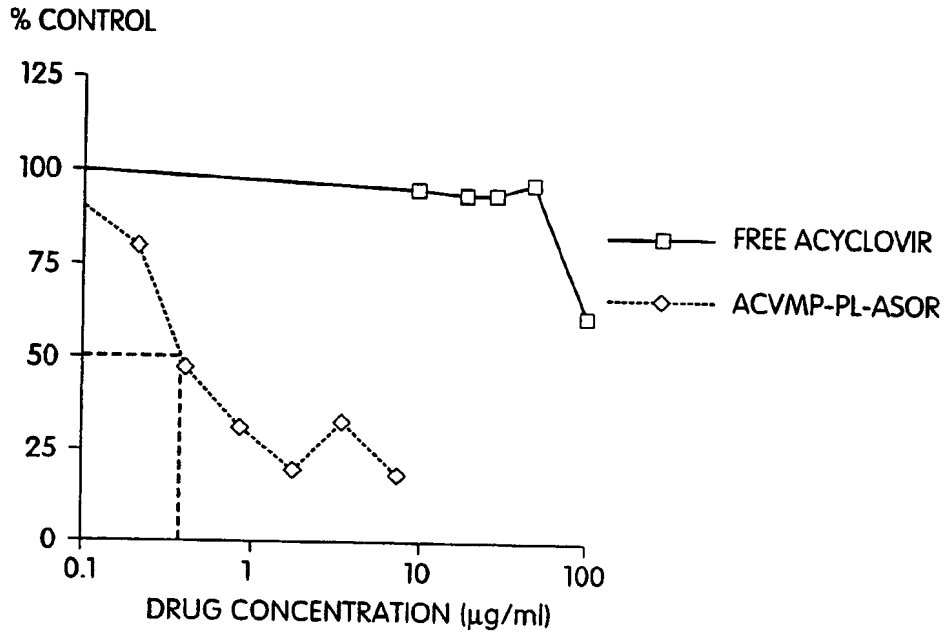
% CONTROL
125
100
75
50
25
0
0.1
1
10
100
DRUG CONCENTRATION (µg/ml)
FREE ACYCLOVIR
ACVMP-PL-ASOR

Fig. 3

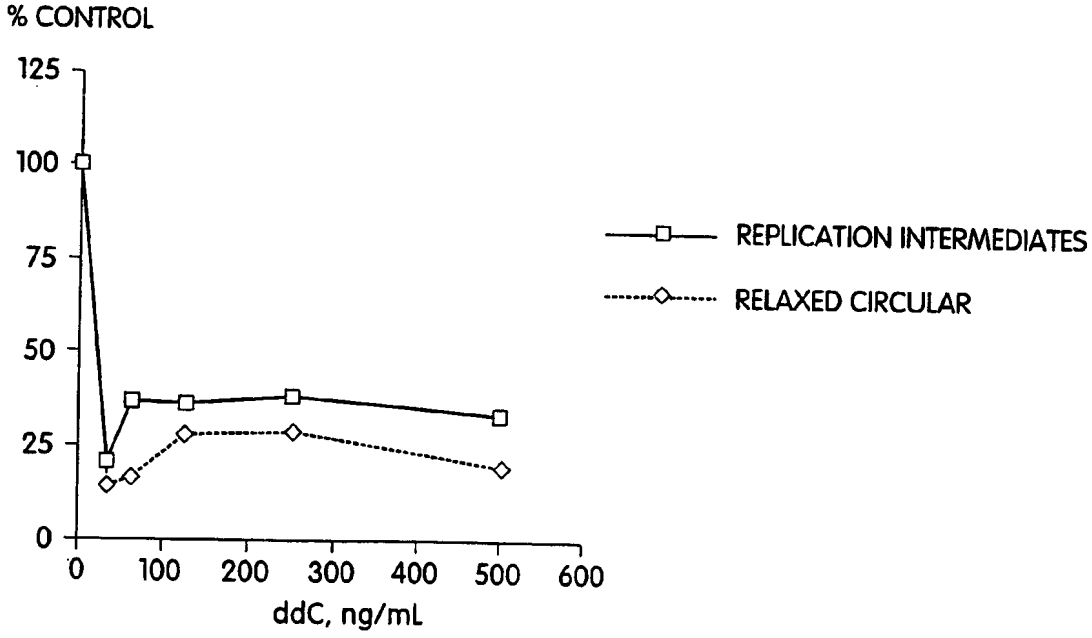
% CONTROL
125
100
75
50
25
0
0
100
200
300
400
500
600
ddC, ng/mL
REPLICATION INTERMEDIATES
RELAXED CIRCULAR

Fig. 4